Characterisation of cuticular mutants in *Arabidopsis thaliana*

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1. Introduction

When plants conquered land around 500 million years ago, they acquired a protection against dangers in the aerial environment. As plants are not mobile, the protection had to be effective and rapidly inducible. Specific dangers for land plants can be divided into two categories: abiotic and biotic stresses. Among others biotic stresses consist of attacks by various viral and fungal pathogens for which plants developed various defence systems. Abiotic stresses include water stress, salt stress, intoxication and UV irradiation. As a strategy to withstand these various stresses plants developed a protective coating, which only made the move from aquatic to terrestrial environment possible. A characteristic feature of land plants is the deposition of a protective layer on top of the outermost cell wall on all aerial organs. This protective layer is called cuticle (Fig.1). It mainly consists of cutin, cutan and waxes. Cutin is a polyester containing hydroxylated and epoxylated long chain fatty acids while cutan is a very thin fraction that is non-hydrolysable and not yet characterised. Cuticular waxes are found on the outermost side of the cuticle. They consist mainly of long chain fatty acids, their esters, alcohols, aldehydes and ketones which form different forms of crystals. In addition to the cell wall, the cuticle provides a barrier against invasions from the external environment.

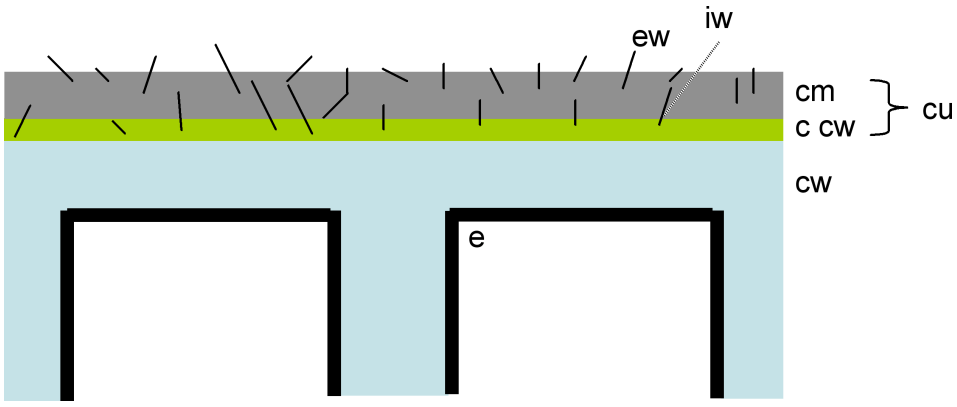

Fig.1. Classical model of the cuticle.
The cuticle comprises the cuticle membrane and the cutinized portion of the cell wall. It covers the underlying cell wall that tops the epidermis cells. Epicuticular waxes are found on the outer extremity and they form different crystal arrangements and are imbeded in the cuticle. (e-epidermis, cm-cuticular membrane, cw-cell wall, c cw-cutinised cell wall, cu-cuticle, ew-epicuticular waxes, iw-intracuticular waxes)

1.1. Biosynthesis of long chain fatty acids

Fatty acids with more than 18 carbon atoms are termed very long chain fatty acids (VLCFA) and they have a wide range of pivotal functions (Leonard et al., 2004). The majority of VLCFA are destined for the outer surface of the epidermis to become a part of the cuticle and wax layer. In plants, VLCFAs are synthesised in the epidermis by the microsomal fatty acid elongation (FAE) system. In this complex, units of two carbon atoms of malonyl-CoA are attached to preexisting C16 or C18 fatty acids that were produced de novo in the plastids by the fatty acid synthase (FAS) pathway. The enzymatic reaction in one round in the FAE complex consists of 4 steps. Firstly the malonyl-CoA is condensated with a long-chain acyl-CoA by a β-keto-acyl-synthetase. Secondly, the product from the first step is reduced to β-hydroxyacyl-CoA. In the third step the product from step two is dehydrated to an enoyl-CoA to be then reduced in the fourth step, resulting in the elongated acyl-CoA. The whole process is termed elongation. Multiple genes and the corresponding mutants have been identified in this pathway. Mutations in those genes inhibited wax formation or accumulation of seed triacylglycerols (Fiebig et al., 2000; Hooker et al., 2002; Millar et al., 1997).

In the *Arabidopsis thaliana* genome, 21 β-ketoacyl-synthetase-like genes or "elongases" have been identified (Lechelt-Kunze et al., 2003; Kunst et al., 2003). The variety of elongases can be explained by the requirement of tissue specific functions and substrate specificities and that elongases produce different products at different developmental stages. Substrates can differ by chain length or saturation state. Several examples are described in the literature. The *FAE1* gene is expressed in seeds and the *fae1* mutant yields a change in the chain length of seed storage lipids but no wax phenotype. *FAE1* is part of the VLCFA elongation complex in the membranes of the endoplasmic reticulum (ER) (James and Dooner, 1991; Kunst et al., 1992; James et al., 1995; Millar and Kunst, 1997). The major β-keto-acyl-synthetase involved in wax biosynthesis in Arabidopsis is CER6 (Millar et al., 1999; Hooker et al., 2002). FIDDLEHEAD (FDH), a homologue to *FAE1* and *CER6*, is putatively involved in cutin biosynthesis. *FDH* is expressed in the epidermis of young organs (Yephremov et al., 1999). The *fdh* mutant exhibits a strong organ fusion

phenotype, suggesting that the cuticle may be affected, but the function of the enzyme is not yet understood.

To produce wax and cutin precursors, the VLCFAs undergo further reactions after the elongation but the pathways and locations are not completely resolved yet.

1.2. Mutants with a defect in cutin biosynthesis

Several genes have been cloned that are involved in the formation of the cuticle in *Arabidopsis thaliana* (Yephremov and Schreiber, 2005). Some of them seem to encode enzymes playing a role in the lipid metabolism: *FDH*, *LCR*, *WAX2/YRE*, *LACS2*, *ACC1*, and *ATT1* (Yephremov et al., 1999; Pruitt et al., 2000; Wellesen et al., 2001; Chen et al., 2003; Kurata et al., 2003; Baud et al., 2004; Schnurr et al., 2004; Xiao et al., 2004). However their functions in the cutin biosynthesis still need to be defined.

In 1998, Lolle et al reported the isolation and characterisation of 29 independently derived mutants with mutations causing organ fusions in Arabidopsis. The mutants could be divided into complementation groups corresponding to nine loci (*airhead*, *bulkhead*, *conehead*, *deadhead*, *cer10*, *fiddlehead*, *hothead*, *pothead* and *thunderhead*). The *hothead* gene was later mapped by Krolikowski et al (2003) to the location of *ADHESION OF CALYX EDGES* (*ACE*) that was briefly described previously (Nakatane et al., 1998). In a phylogenetic analysis Krolikowski et al (2003) discovered that there are seven homologues of *ACE/HTH* which they called *HOTHEAD-LIKE 1-7* sharing amino acid identity in the range of 40 to 60 %. Expression analysis was carried out on *HTH* by Krolikowski et al (2003) using RT-PCR, and they showed that the *HTH* gene is highly expressed in leaves, stem and inflorescences but also expressed in roots. In the same article, the authors reported in situ hybridisation studies in which the hybridisation of the *HTH* probe does not only occur in the epidermis but also in all sub-epidermal tissues and all floral organs. The author concluded that *HTH* has a general function and is not involved solely in cuticle synthesis. Sequence similarity searches showed that *ACE/HTH* contains GMC oxidoreductase domains and that the closest homologue was characterized as a mandelonitrile lyase (MDL). MDL is one of four enzymes responsible for the catabolism of seed macerates in rosaceous stone fruits (Hu et al., 1999). MDL

catalyses the last step in the degradation of diglucosides to HCN, glucose and benzaldehyde (Poulton et al., 1993). Despite these sequence similarities the function of ACE/HTH remains unknown.

Moreover, Lolle et al (2005) claimed that the fundamental tenet of classical mendelian genetics is not true for the *hth* mutant. The authors reported that homozygous *hth* mutants after self-fertilisation revert to heterozygous plants with a frequency of 10^{-1} to 10^{-2} revertants per chromosome per generation. Approximately 10 % of the progeny were revertants. They proposed that this process is template-directed as the sequence change is the very specific reversion to the wt sequence. The authors' model stated that stable RNA, likely in a double stranded form, can be replicated and transmitted over generations, and is likely being inherited via pollen. In this way it can restore the sequence information cached from previous generations. This phenomenon can be observed on other mutants but can only be seen in the *hth* background as these mutants are put under an indirect stress due to the absence of the HTH gene product. Other scientists tried to explain the *hth* phenomenon differently (Comai and Cartwright, 2005; Chaudhury, 2005). Comai and Cartwright state that there are two components that lead to the reversion of the *HTH* mutations: firstly that the mutant accumulates toxic or rather mutagenic compounds which lead to a higher variation rate in its genome and secondly that there is an early selection for the *HTH* revertant alleles as there is no accumulation of toxic compounds. Additionally, early revertants have fewer deleterious mutations in the genome that is the reason why they are detectable. To explain why small fragments of RNA are able to repair the *hth* and other mutations, Chaudhury (2005) proposed that very short homologous sequences in the genome are responsible for the production of these RNAs and that the mismatches generated by these RNAs are normally corrected by mismatch repair. Ray (2005) stated that extra chromosomal DNA fragments are archived in a way that they cannot be detected by DNA hybridisation or PCR. These fragments might then be restricted to meristematic cells only and therefore be present in undetectable concentrations in classical experiments. However, the question how the non mendelian passing on of information in *hth* works still needs to be unraveled as well as the molecular function of the *HTH* gene.

Other genes appeared to be indirectly involved in the biosynthesis of the cuticle components based on sequence similarities and expression patterns: *ALE1*, *ACR4*, *WIN1/SHN* and *PAS2/PAP* (Tanaka et al., 2001, 2002; Bellec et al., 2002; Haberer et al., 2002; Aharoni et al., 2004; Broun et al., 2004; Watanabe et al., 2004). Phenotypes of these mutants are pleiotropic including cuticular defects, growth abnormalities, an increased sensitivity to chemicals, an altered resistance to pathogens, occasional distorted cell differentiation and cell death (Yephremov and Schreiber, 2005). Two Arabidopsis mutants, one expressing a fungal cuntinase from *Fusarium solani* f sp. pisi a (Sieber et al., 2000) and the *lcr* mutant that carries a mutation in cytochrome P450 *CYP86A8* (Wellesen et al., 2001), exhibited a cell wall phenotype besides the cuticle phenotype. They demonstrated morphological irregularities in the shape of organs and single cells similar to those in known cell wall mutants as *radial swollen* (*rsw*) (Williamson et al., 2001). More cuticular mutants were identified in different collections of *A. thaliana* lines (Lolle et al., 1998; Tanaka et al., 2004) and not all exhibit organ fusion.

Tanaka et al (2004) screened for mutants with an accelerated penetration phenotype. The author applied toluidine blue solution to leaves. When this cationic dye penetrates the cuticle and binds to the anionic polysaccharides and pectins of the cell wall it can no longer be washed out and the tissue turns blue. Tanaka applied toluidine blue staining to a selection of mutagenised plants and selected those in which the dye stained the cell wall faster than in the wild type (Tanaka et al., 2004). From this selection of the *permeable leaves* mutants (pel), *pel3* was mapped and molecularly identified by Tanaka et al (unpublished data, personal communication). The *pel3* gene shows homology to transferases but its function remains unknown. Despite the accelerated penetration of the dye through the cuticle, the *pel3* mutant exhibits no obvious cuticle phenotype on leaves but occasionally strong deformations and fusions in the flower. On approximately every second plant the inflorescences show fused petals and fusions involving multiple organs. Ubiquititous PEL3 gene expression was found by using ‚genevestigator' (Zimmermann et al. 2005), in shoots, in the flower (petal, sepal, siliques, seeds-only stage 6), in young rosettes.

Another fusion mutant is *bodyguard* (*bdg*). The lines *bdg-1* and *bdg-2* mutants were selected from an Arabidopsis population mutagenized with the *Zea mays*

transposon *Enhancer/Supressor*-mutator (En/Spm) (Kurdyukov et al., 2006). The *bdg* mutant has a strong cuticular phenotype displaying leaf fusions. This can be seen on transmission electron microscopy images (TEM) in the formation of multilayered structures instead of a thin-layered cuticle and patches with no cuticle at all (Kurdyukov et al., 2006). The *BDG* gene has been cloned by transposon tagging. It encodes an α/β-hydrolase fold containing protein of unknown function. It has been shown that *BDG* is expressed in the epidermis. This expression pattern has been assesed by GFP and GUS-fusions, in situ hybridisation analysis and also with immunolocalisation experiments in which the BDG protein was detected in the inner part of the outer epidermal cell wall (Kurdyukov et al., 2006).

The connection between the different cuticle phenotypes is not yet clear and further investigations will be needed to completely enlighten the anabolism of cutin precursors, their transport, the mechanism of interconnection in and on the cell wall and the effects of different irregularities of the cuticle.

1.3. Mutants with a defect in wax biosynthesis

Generally, waxes consist of aldehydes, primary and secondary alcohols, alkanes, ketones and esters that are derived from saturated VLCFAs with predominant chain lengths from 20 to 34 carbons (Walton, 1990; Kunst and Samuels, 2003). Based on the composition of the waxes, a general model has been developed for the biosynthesis of the wax components (Fig.2) (Kunst and Samuels, 2003). The VLCFAs with even numbered chain lengths of more than 20 carbon atoms, coming from the FAE complexes are processed through two different pathways: the acyl reduction pathway and the decarbonylation pathway (Fig.2). In the acyl reduction pathway the VLCFAs are reduced to primary alcohols by acyl reductases. As intermediates of this reaction VLC aldehydes are formed which stay bound to the enzyme and do not appear in a free form. In the last step of this reaction the primary alcohols and the VLCFAs form esters with the help of acyl-CoA:alcohol-acyltransferases. In the decarbonylation pathway the VLCFAs are reduced to aldehydes by an acyl-CoA-reductase that is different to the one in the acyl reduction pathway. These acyl-CoA-reductases release the aldehydes and these free aldehydes are substrate for the next reaction. In this next step aldehyde

dehydrogenases catalyse the production of alkanes by cleaving carbon monoxide from the aldehyde. These alkanes with an odd number of C-atoms in their carbon chains are hydroxylated and further oxidized to secondary alcohols or ketones. Known enzymes involved in these pathways and in the transport and regulation of these pathways are listed in Table.1.

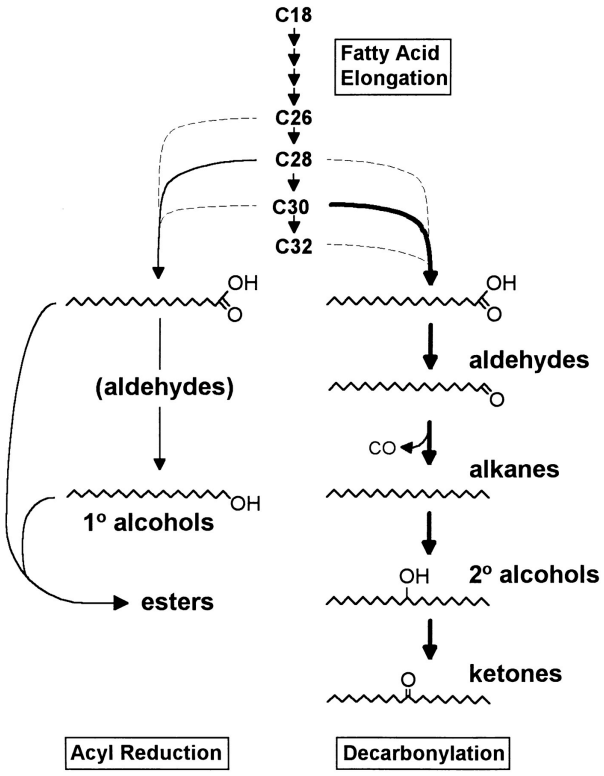

Figure 2. Putative pathway for biosynthesis of waxes (Millar et al., 1999).

In Arabidopsis, 22 loci are considered to be involved in the wax biosynthesis (Dellaert et al., 1979, Koorneef et al., 1989). The mutants accumulate less wax, exhibit greener stems, siliques and occasionally greener leaves when compared to the wild type. They are called *ecériferum* (*cer*) and were produced by irradiation with x-ray and EMS mutagenesis (Dellaert et al., 1979; Koorneef et al., 1989). SEM was performed on the stems of the mutants and revealed differences in the wax layer in comparison to the wild type. Further, it was reported that some *cer* mutants have a reduced size and reduced fertility, especially *cer10* and particularly some mutants under low humidity conditions (*cer1, cer3* and *cer6*) but it was not tested further. In 1993, Preuss et al showed that this reduced fertility phenotype is caused by the erosion of the pollen tryphine layer owing to the absence of pollen surface waxes.

Other model plants revealed different numbers of loci involved in wax biosynthesis. In barley 84 loci are known to regulate the wax biosynthesis (Sogaard and von Wettstein-Knowles, 1987 and von Wettstein-Knowles, 1993). In maize, 20 loci were found to be involved in wax production and transport. Due to the glossy appearance of their mutants they are termed glossy 1-20 (Schnable et al., 1994). Some wax mutants in maize have been characterised in more detail, for example: *glossy1* (Sturaro et al., 2005), *ad1* and *cr4*.

In *Oryza sativa* no wax mutants have been reported yet although wax analyses have been reported in rice. These were mainly conducted on different rice varieties as the wax load has been reported to be an adaptive characteristic for the drought resistance (O'Toole and Cruz, 1983). Among rather usual wax components, large amounts of aldehydes have been mentioned to be contained in the rice waxes (Welker et al., 1998; Haas et al., 2001). These were reported to occur in a polymeric form as aggregates of "aldehyde clusters" (Welker et al., 1998; Haas et al., 2001).

Overall, only some of the Arabidopsis *cer* genes have been identified (Table 1).

Table 1. CER genes with known functions

	Function	**Refercence**
CER1	decarbonylase	Aarts et al., 1995
CER2	putative involvement in regulation of elongation	Xia et al., 1997
CER3	protein degradation	Hannoufa et al., 1996 Eisner et al., 1998
CER4	alcohol forming fatty acyl-CoA reductase	Kunst, L., 2005
CER5	ABC-transporter for wax export	Pighin et al., 2004
CER6/CUT1	β-ketoacyl synthetase, condensing enzyme of the elongase complex	Millar et al., 1999 and Hooker et al., 2002
CER10	enoyl CoA reductase	Zheng et al., 2005
WIN1/SHINE1	ethylene response factor-type transcription factor	Aharoni et al., 2004 and Broun et al., 2004

1.4. Mutants with a defect in wax and cutin biosynthesis

A few mutants have been reported to have both, an obvious wax deficit plus an organ fusion phenotype. In Arabidopsis, these mutants were *cer10, cer13, wax1* and *wax2* (Jenks et al., 1996), *deadhead* (Lolle et al., 1998) and in *Z.mays adherent1* (Sinha and Lynch, 1998).

Zheng et al (2005) identified *cer10* as At3g55360, which codes for an enoyl CoA reductase (ECR). In a reverse genetic approach, the authors found Arabidopsis lines with T-DNA insertions in the *ECR* gene exhibiting the same phenotype as *cer10*. In a complementation test it was demonstrated that *ECR* and *CER10* are allelic. Green fluorescent protein-*ECR* revealed the reticulate network typical for the localisation in the ER in all examined organs, suggesting that ECR was especially localised in the ER membrane. This is similar to the location of TSC13p, the enoyl CoA reductase in yeast cells. Kohlwein et al (2001) suggested that TSC13p is a component of the ER and showed an enrichment in the vacuole-nuclear envelope sections which was similar to the location of the yeast ELO proteins, the condensing enzymes of the fatty acid elongation complex in yeast. The enoyl CoA reductase is supposed to catalyse the final reaction of VLCFA biosynthesis. In yeast, *TSC13p* has been identified to code for such an enzyme. It has been demonstrated that it is essential for yeast viability and that it catalyses a step in the fatty acid elongation cycle for acyl-CoA substrates of all chain lengths (Kohlwein et al., 2001). The orthologue was found in *A. thaliana* to be At3g55360 which was annotated as a gene similar to the mammalian steroid 5-alpha-reductase. Five homologues exist in the Arabidopsis genome, three of them are closely related to *CER10/ECR* (Costaglioli et al., 2005). Expression studies of the five homologues were carried out in eight days old *A. thaliana* seedlings, since Arabidopsis leaves carry the highest wax load per area unit at that age. Expression analysis revealed significant levels of transcript of At3g55360 and At5g16010 only. The expression of At5g16010 was significantly higher than of At3g55360 suggesting that the corresponding protein could play an important role at this stage of development (Costaglioli et al., 2005). Analyses of the wax composition on leaves and stems on *cer* mutants revealed that there was a shift of wax components to the longer chain lengths in *cer10*. In leaves this was mainly due to a reduction of the C29 alkane and the increase of C30 primary alcohol (Rashotte et al., 2001). On stems, a similar pattern with reduced C29 components

and increased C30 primary alcohol levels was detected. The authors proposed a putative function of *cer10* in the conversion of the C30 fatty acid to C30 aldehyde. The discrepancy between the conclusion based on these wax analyses and the function of the known gene remains elusive but it may be due to the redundancy of the enoyl CoA reductase in Arabidopsis and compensation for the lack of *CER10/ECR* by the means of other metabolic pathways. Lolle et al. (1998) assayed the permeability of the cuticle for chlorphyll in *cer10* using the chlorophyll leaching test. This test is based on extraction of whole tissues with ethanol and photometrical estimation of the extracted chlorophyll. The chlorophyll in *cer10* leached out more rapidly than in the wild type and similar to other fusion mutants such as *fdh*. Another phenotype of *cer10* was characterized by the reduction of cell size of epidermis cells on leaves and the less pronounced lobe structures of epidermal cells (Zheng et al., 2005). The *Nicotiana benthamiana ECR* (NbECR) was silenced by using virus-induced gene silencing (VIGS) (Park et al., 2005). This caused formation of necrotic lesions on young leaves and an abnormal leaf development with wrinkled leaves and irregularities in the shape. The leaves exhibited general defence responses like accumulation of large amounts of callose and expression of numerous defence genes (e.g.*PR1a, PR1b*). Analysis of saturated fatty acids revealed a reduction in fatty acids longer than C18 in plants with a disrupted *NbECR* expression. Also, these plants showed highly abnormal plasma and thylakoid membranes. The progression of the degeneration was indicated by loops and irregular surfaces of the membranes leading to the desintegration of the cell. It was proposed that the *NbECR* is more essential in *N. benthamiana* and provides most of the enoyl-CoA reductase activity for VLCFA synthesis whereas in Arabidopsis the loss of *CER10/ECR* can be partially complemented by other enzymes.

Another wax mutant that has been reported to show occasional organ fusions is *cer13*. Wax analysis has been carried out by Rashotte et al (2001). The leaves of *cer13* show a general reduction in the wax load but without a change in the wax profile. On *cer13* stems, a measurable reduction in all C29 components and increase in C31 alkanes and C30 primary alcohols was detected, meaning chain length distribution is shifted from shorter to longer chain lengths when compared to the wild type. The mutant *cer13* resembles the wax phenotype of *cer3* and *cer7* (Jenks et al., 1995). For CER13 the authors proposed an involvement in the reduction of C30 fatty

acids to C30 aldehydes because the blockage of that pathway could explain the increase in chain length in *cer13* wax components. An accumulation of C30 fatty acids leads to an increase in the reduction of these fatty acids to the C30 primary alcohol or the further elongated C32 fatty acids. These C32 fatty acids could be reduced to C32 aldehydes and decarbonylated C31 alkanes. The results of the wax analysis on stems supported this hypothesis. Still another possibility could be an involvement of CER13 in the release of the C30 fatty acid from its elongation complex. For all possible involvements in the wax biosynthesis there is more than one gene involved in the same process as CER13 because the mutation does not cause a complete loss of C30 aldehyde or downstream products.

The *wax2* mutant was described in Kurata et al (2003) with the name *yore-yore* (*yre*). *Yre-1* was isolated in an Arabidopsis population of T-DNA insertion lines that was screened for mutants defective in cell differentiation. This allele of *wax2* exhibited smaller trichomes that showed a normal distribution and normal branching. It showed a wax deficiency on stems which resembled the *cer* phenotype. *Yre-1* exhibited reduced male sterility which could be recovered under high-humidity conditions. The abscission of siliques was delayed in *yre-1*. Additionally tissue adhesion was observed in leaves, flower buds, and in other flower organs.

Chen et al (2003) described the identification and characterisation of *wax2* in a collection of 30000 insertion mutants, which they screened to identify lines with reduced visible glaucousness of the inflorescence stem. Further inspection of *wax2* revealed that it did not only have a wax phenotype but it also exhibited organ fusions. TEM of the cuticle showed a thicker but less opaque cuticle (151,7 nm in *wax2* and 111,2 nm in wild type). The authors described a weight loss of 20,2 % of $ZnCl_2$-isolated cuticles and *wax2* stem cuticles compared to wild type. Wax crystals were reduced on *wax2* as seen on SEM pictures, and the wax analysis showed a quantitative reduction of waxes on leaves (80,3 %) and stems (78,3 %) in *wax2* when compared to the wild type. Organ fusions were reported for most aerial organs of *wax2*: leaves, sepals, flower buds, sepals and stems. Fusions only occurred within the same plant but never between two different plants. The male fertility was impaired but could be restored under high humidity conditions. Visual inspection of the epidermis revealed a different stomatal index for *wax2*: on the abaxial side the

stomatal index in *wax2* was reduced by 17,4 % and on the adaxial side there was a reduction of 16 % when compared to the wild type. Further changes in the epidermis were a reduced stomatal index and increased density of pavement cells. Permeability changes were assayed on *wax2* and the corresponding wild type by a chlorophyll leaching test that showed a higher rate of chlorophyll leaching in *wax2* than in the wild type. The application of two herbicides (acifluorfen and paraquat) caused greater foliar injury in the *wax2* mutant than in the wild type. Discoloration and wilting occurred more readily in *wax2* which was likely due to a faster permeation of the herbicide molecules through the cuticle and the epidermis into the underlying tissue. Not only these molecules could more readily permeate the cuticle but also the water transpiration rate was higher in *wax2* in the dark as well as in the daylight, which according to the authors, excludes a defect in stomata closure.

As some wax phenotypes are not found without biochemical analysis, more fusion mutants with wax phenotypes will be found when their wax composition is analysed. One example is the *bdg* mutant which shows a severe fusion phenotype and no evident wax distortion. But wax analysis reveals that there is a greater accumulation of wax components (Kurdyukov et al., 2006). Apart from these known mutants with a fusion and wax phenotype, there are very likely others.

1.5. Analysing wax and cutin

The biochemical analysis of waxes is well established and usually performed using gas chromatography coupled to either a mass spectrometer (GC-MS) or with a flame ionisation detector (GC-FID). Hydroxyl containing compounds are rather unstable, cannot be well separated in GC conditions and need to be transformed into inert and stable derivatives, which lead to unequivocal mass spectra (Seibl, 1970). One frequently applied method for the derivatisation of wax components is the transformation of the hydroxylated compounds into their trimethylsilyl-ethers and -esters with N,O-bis(trimethylsilyl)trifluoracetamid (BSTFA).

Cutin has been analysed especially from plants with thick cuticles (Kolatakutty, 1981, 2001; Holloway, 1982). For the analysis, the celluloses had to be removed from the peeled cuticles first. This was achieved by digestion with pectinases and

cellulases. Then the cuticles were extracted with methanol/chloroform to extract all soluble lipids such as epicuticular and intracuticular waxes. The next step involved the depolymerisation by procedures cleaving ester bonds. Depolymerisation steps could be carried out using alkaline hydrolysis, transesterification with methanol containing boron trifluoride or sodium methoxide or by using a reductive cleavage with lithium aluminium hydride (Walton and Kolattukudy, 1972; Kolattukudy, 1981). The liberated cutin monomers could than be derivatised similar as the waxes and also analysed by GC-MS and –FID to identify the cutin monomers by their characteristic fragmentation patterns (Walton and Kolattukudy, 1972) and determine quantitative amounts. Classical cutin components are fatty acids (C16 and C18), hydroxylated fatty acids (C16 and C18, saturated and unsaturated), di-hydroxy fatty acids (C18, saturated), hydroxy-epoxy fatty acid (C18) and tri-hydroxy fatty acids (C18). Minor amounts of VLCFAs are fatty alcohols, aldehydes, ketones, diacids as well as hydroxycinnamic acids.

The application of known methods for the analysis of the very thin and fragile Arabidopsis cutin was not successful for a long time. Recent advances showed a novel approach to analyse cutin in *A. thaliana* (Xiao et al., 2004; Bonatventure et al., 2004; Franke et al., 2005). Xiao et al. (2004) described a method with which the stem cuticle was manually peeled of using forceps after estimating the area of the cuticle. It was refluxed in chloroform/methanol (1:1) for 24 hours to get rid of all soluble material from the cuticle and then depolymerised the with 14% BF_3 in anhydrous methanol to produce methyl esters which could then be extracted with diethyl ether as already described in Riederer and Schönherr (1986). Derivatisation was carried out using N, O-bis (trimethylsilyl) trifluoroacetamide and incubated for 30 min at 70°C. Samples were analysed on GC-FID. The analysis revealed for the Columbia wild type C16-diacid (49,3 %) and C16-10,16-dihydroxy-acid (16,4 %) as major components. Other compounds in the Columbia cuticle included: C18-diacid (11,8 %), C16-7 hydroxy-diacid (11,3 %) C17-9/10 hydroxy-acid (3,9 %), C16-w hydroxy-acid (3,2 %) and C15-9 hydroxy-acid (2,6 %). The total amount of cutin components on the Columbia stem surface was 0,491 µg cm^{-2} (+/- 0,0082).

A different approach in analysing *A. thaliana* cuticles was published by Bonaventura *et al* in 2004. In these analyses whole *A. thaliana* tissues were used

and extracted prior to the depolymerisation instead of the preparation of cuticular membranes. The ester bonds were cleaved by hydrogenolysis with lithium aluminium hydride ($LiAlH_4$) and the carboxy groups were reduced to their primary alcohols. Other functional groups, such as aldehydes, ketones and oxiranes were reduced to their primary or secondary alcohols. Subsequent analysis was carried using GC-MS and revealed C16 and C18 polyhydroxy alkanes and alkenes as main hydrogenolysate products. The most abundant product in leaves was C18-(2)-1,18 diol. The polyester composition on stems resembleed the same components as on the leave extracted cuticles. To discriminate between the origin of the different hydroxyl groups the authors employed an isotopic labelling by deuteriolysis with lithium aluminium deuteride ($LiAlD_4$) to the extracted tissues. With this method it was possible to distinguish from which part of the molecule the polyols derived from e.g. the C18-(2)-1,18 diol derives primarily from C18-(2)-diacid, with smaller amounts arising from the corresponding ω-hydroxylated fatty acid and very small amount from the ω-oxo-fatty acid and 1, ω-diol. Bonaventure et al (2004) applied another method on extracted leaves to verify the results of the hydrogenolysis and the deuteriolysis. They used sodium methoxide ($NaOCH_3$) in methanol for transmethylation of the cutin esters with subsequent silylation of the products. The major product in the leaf cuticle was the methylated C18-(2)-diacid and confirmed the results from their first analysis. By comparison of the two methods they found out that the transmethylation method produced a more variable monomer content (up to 50 % variation) compared to the hydrogenolysis and deuteriolysis. To confirm that the analysed components were from the cuticle and not from underlying tissues the authors depolymerised epidermal peels from Arabidopsis stems without the extraction of soluble lipids. The result was similar to that obtained with stem and leaf residues but the waxes were obviously also seen. This indicates that the dicarboxylic fatty acids and other components analysed on totally extracted stems were primarily part of the polyester in the epidermis. The remains of the stem without the epidermis was also analysed and did not produce any detectable C16 or C18 polyhydroxylated aliphatic compounds when analyzed by GC indicating that all components mentioned above are associated with the epidermis.

The results for the cutin composition of Bonaventure et al (2004) contradict those of Xiao et al. (2004), who describe C16-diacids as the major component of the

Arabidopsis cutin. Whereas Bonaventure's analysis revealed C18-(2)-diacid as the major cutin component. Both authors analysed cutin of stems: this discrepancy may therefore be due to the different ecotypes they analysed. Bonaventure analysed the Arabidopsis Wassilewskija-2 ecotype and Xiao Columbia.

The cutin biosynthetic pathway seemed to include hydroxylation and epoxidation steps (Kolatakutty, 2001). But the genes encoding enzymes in this pathway have not yet been unequivocally identified (Kolatakutty, 2001; Nawrath, 2002). Arabidopsis is a good model plant to study genes involved in the cutin biosynthesis as genetic and biochemical approaches can be combined. The screening for cuticular mutants in Arabidopsis is important to search for candidate genes that are involved in cutin biosynthesis. For mutant screening, the application of dyes to measure a change in the penetration rate in cuticular mutants (Tanaka et al., 2004) is the method of choice. Some cuticular mutants can be found as they show different responses to pathogens (Xiao et al., 2004; Nawrath et al., 2005) or organ fusions (Lolle et al., 1998). It is now possible to use reverse genetics and search for knock-out mutants in family members of genes that were already identified by forward mutant screening. Methods to analyse mutants with a putative defect in cuticle biosynthesis and detect the consequent defects in *A. thaliana* include checking for the obvious organ fusion phenotype, chlorophyll leaching, water evaporation tests, TEM and SEM imaging, the weighing of cuticles, staining with toluidine blue, application of herbicides, the test for pollen germination on non reproductive organs and the test for the response to the application of pathogens. Tests for putative secondary effects include checking of morphological features such as measurement of the stomatal index, density of pavement cells or analyses on trichomes. The cutin composition analysis suggested fatty acid elongation, hydroxylation and epoxidation steps to be involved in the cutin biosynthesis but the localisation, the active sites and activities of putative enzymes that play a role in the cuticle biosynthesis need to be further scrutinized. Methods of choice include expression analyses (GFP/GUS promoter fusions, in situ hybridisation analysis), introduction of point mutations to check specific functions of the enzyme and expression with different promoters (e.g. epidermis specific, seed specific, inducible by antibiotics or ubiquitous expression with 35S). With the help of these tools, the detailed roles of the enzymes involved in the pathways will be elucidated.

1.6. Aim of the work

Different approaches have been taken to understand cutin biosynthesis and the function of the cutin monomers. However, many questions still remain unsolved. Analysis of cutin lead to the knowledge of the composition on a wide variety of plants but only the combination of this knowledge with genetic approaches using model plants such as Arabidopsis, its mutants and gene knock-outs, it will be possible to fully understand the mechanism of the synthesis of cutin components, the interaction of lipids and their role in defence and signalling.

The work aimed to develop and verify an appropriate method for the analysis of cutin in Arabidopsis, which can subsequently be applied to all plants with very thin cuticles. The method should be applicable to different mutants and to wild types grown under specific conditions to gain insight into the cutin composition of different lines, developmental stages or under specific stress conditions. In the second part of the project known fusion mutants were further investigated. Two of the *cer* mutants that exhibit the fusion phenotype besides the reduction in waxes, namely *cer10* and *cer13*, were chosen for further characterisation. Both genes seem to play a role in the pathways and their functions had to be analysed based on their phenotypic description and biochemical analyses. With the focus on two mutants that exhibit both, a wax and a cuticular phenotype, more information about these mutants might reveal deeper insight to both pathways and their interconnection and effects on plant development and morphology.

2. Materials and Method

2.1. Chemicals, enzymes, media, buffers and solutions

Chemicals used in this work were obtained from either of the following companies: Bio-Rad (USA), Biozym (Hess. Oldendorf), Roche (Mannheim), Difco Lab. (USA), Fluka (Switzerland), Gibco-BRL (Neu-Isenburg), Merck (Darmstadt), Promega (Heidelberg), Qiagen (Hilden), Riedel de Häen (Hannover), Roth (Karlsruhe), Sigma (München). The degree of purity was “for analysis”.

Restriction enzymes were obtained from the companies New England Biolabs (Schwalmbach), Roche (Mannheim), Stratagene (Heidelberg) and Fernentas (St. Leon-Rot). The digestions were done as suggested by the manufacturer.

Media, buffers and solutions were prepared as specified in Sambrook et al (1989).

2.2. Bacteria and Plasmids

E.coli strain:
DH5α: Genotype: supE44 DlacU169 hsdR17, recA1, endA1, gyrA96, thi-1, relA1, F (Hanahan, 1983)

Agrobacterium tumefaciens:
GV3101 pMP90RK (Koncz et al., 1990)

Plasmids
pDONR™ 201 (Invitrogen, Heidelberg) kan^R
pAM-PAT 35S GW Terminator (Bekir Uelker and Imre E. Somssich) amp_R

2.1.1. Preparation of chemo-competent *E. coli* cells (Hanahan, 1983)

All steps in this experiment were performed at 4°C. The volume of 5 ml of an over night culture of *E.coli* strain DH5α was added to pre-heated 100 ml of LB and kept on shaking at 37°C to achieve the bacterial growth to the OD_{600} of 0.5-0.6. The bacteria

were centrifuged at 5000 x g for 10 minutes at 4°C. After discarding the supernatant, the pellet was gently re-suspended in 30 ml ice-cold TFBI solution. After the second step of centrifugation, the pellet was resuspended in ice-cold TFBII solution.. Eppendorf reaction tubes of 1,5 ml containing 50 µl aliquots of cells were frozen in liquid nitrogen and stored at -80°C until use.

2.1.2. Transformation of chemo-competent *E. coli* cells

For every transformation, one aliquot of chemo-competent cells was thawed on ice. For the Gateway recombination reaction, the entire reaction mixture (as described in 2.6: 6 µl or 5 µl, respectively) was added to an aliquot of *E. coli* DH5α cells. The cells were incubated on ice for 30 minutes. The mixture was heat-shocked for 30 seconds at 42°C and again incubated on ice for 2 minutes. The volume of 900 µl of SOC medium was immediately added to the Eppendorf tube and incubated at 37°C for 1 hour with continuous shaking at 950 rpm. A fraction (~ 150-200 µl) of the transformation mixture was plated out onto selection media plates. Transformed colonies were isolated.

2.1.3. Preparation of electrocompetent *A. tumefaciens*

The *Agrobacterium* strain GV3101 was used for the described transformation. The strain has a C58C1 chromosomal background is marked by a rifampicin resistance mutation. It carries pMP90, a helper Ti plasmid, marked with a gentamycin resistance encoding virulence functions for T-DNA transfer from *Agrobacterium* to plant cells (Koncz et al., 1990).

A single colony of *A. tumefaciens* was inoculated into 5 ml of YEB medium and grown o/n at 28°C. The o/n culture was used to inoculate 400 ml of YEB medium and grown to A_{600nm}=0.5. Cells were harvested by centrifugation at 5000 rpm and successively resuspended in 200 ml, 100 ml and 10 ml of ice-cold 1 mM Hepes (pH=7.5). Finally cells were resuspended in 800 µl of 1 mM Hepes (pH=7.5) and 10% v/v glycerol, aliquoted and frozen at –70°C.

2.1.4. Electroporation of *A. tumefaciens* cells

An aliquot of frozen electrocompetent *A. tumefaciens* was thawed on ice and mixed with 450 µl of 10% glycerol. The electroporator was set to 25 µF, 2.5 kV and 200 Ω. A single electroporation pulse was given and 1 ml of YEB medium immediately added. After incubation at 28°C for 2 hrs, cells were plated on selective YEB medium with the appropriate antibiotic and incubated for 2 d at 28°C. Transformed colonies were isolated.

2.3. Plant material

Arabidopsis thaliana (L.) Heynh. with the ecotypes of Columbia and *Landsberg erecta* was used in this work.

2.3.1. Cultivation of *A. thaliana*

Plants were raised in the greenhouse under long day (18 hrs day light/8 hrs night) or short day conditions (16 hrs day light/8 hrs night) with a temperature of 17-20°C and a relative humidity of 50-60%.

2.3.2. Cultivation of *O. sativa*

Rice plants were raised in a chamber with a constant temperature of 32°C and under high humidity conditions. From 8 am to 6 pm the light was on and from 6 pm to 8 am it was dark. After the germination of rice seeds the plants were kept on mix of dry sand and soil and on the 31st day after germination the plants are drowned in water up to 20 cm.

2.3.3. In planta transformation of *A. thaliana*

Agrobacterium clones carrying the plasmid were grown in 5 ml of LB medium with gentamycin (25 mg/l) and rifampicin (50 mg/l) o/n at 28°C. The o/n culture was used to inoculate 400 ml of YEB medium and grown for 16-20h. Cells were harvested by a centrifugation step at 5000 rpm and resuspended in 50 ml of 5% sucrose solution. The resuspended culture was brought to A_{600nm} = 0.8 by dilution with 5% sucrose solution. Before transformation Silwet L-77 (500 µl/l) was added to the *A.*

tumefaciens culture. Arabidopsis plants were grown under greenhouse conditions at a density of 9 plants/pot (9 cm diameter). The first emerging stems were cut off to help growth of multiple secondary bolts. Transformation was performed approximately 2 weeks after clipping. The plants were dipped for 30 s into *A. tumefaciens* culture and covered with a plastic hood for 24 hrs to maintain high humidity. After removal of the plastic hoods, plants were kept in the greenhouse until seeds were harvested. The selection of the transformed plants was performed by spraying 7 day old transformants with 0,1% BASTA.

2.3.4. Histochemical staining of *O. sativa*

Whole mount GUS staining was carried out as described in: Arabidopsis: A laboratory manual. X-Gluc (5-bromo-4-chloro-3-indolyl β D-glucuronide (cyclohexamine salt) was used for the histochemical staining of transgenic plants.

2.3.5. Insertionlines

A. thaliana T-DNA insertion lines were obtained from TAIR, GABI Kat (Köln) and INRA (Versailes, France). The *wda1* T-DNA insertion line of *O. sativa* was provided by Gynheung An and Ki-Hong (Pohang, Republic of Korea).

2.3.6. Selection of transgenic plants on grodan mats

This selection was carried out using the protocol of Hadi et al (2001). Plants were selected for sulfadiazin resistance.

2.4. Isolation of genomic DNA using the CTAB method

The CTAB protocol was developed by Murray and Thompson (1980), modified from Rogers and Bendich (1988) and adapted by Rios et al (2002).

2.5. Isolation of plasmids

The “Qiaprep Spin Miniprep Kit” by Qiagen (Hilden) was used for plasmid isolation from *E. coli.*

2.6. Cloning strategies

Plasmids are described in 2.2.

BP reaction

*att*B-PCR Product (50 ng/µl)	1 µl
GATEWAY® BP clonase	1 µl
BP reaction buffer (5x)	1 µl
pDONR™201 vector (50 ng/µl)	1 µl
ddH_2O	1 µl

LR reaction

Entry clone (50 ng/_l)	1 µl
GATEWAY® LR clonase	1 µl
LR reaction buffer (5x)	1 µl
Destination vector (50 ng/_l)	1 µl
ddH_2O	1 µl

Typically, reactions were carried out in 1.5 ml Eppendorf tubes. Reactions were incubated at 25ºC for 12 h (for Gateway cloning), before completely transformed into *E. coli* strain DH5α.

2.7. Plasmid DNA cleavage by digestion with restriction endonucleases

Isolated plasmids were cleaved in diagnostic sites using restriction enzymes in order to confirm the accuracy of obtained construct. For the digestion of plasmid DNA with restriction endonucleases, buffers supplied by manufacturers were used. Cleavage of DNA was performed at recommended optimal temperatures, usually at 37°C. 5-10 U of enzyme were used. Digestion of plasmid DNA and was performed for 1-3 hrs. Enzyme reactions were stopped by heat inactivation of restriction enzymes upon transfer of the restriction mix to 65° for 20 min.

2.8. Isolation of RNA of plants

For the isolation of RNA from plants the “RNeasy Kit” from Qiagen (Hilden) was used with an additional step to digest the DNA on column. RNA concentration and quality was determined by spectrophotometric measurements and agarose gels.

2.9. Semi-quantitative RT-PCR

Semi-quantitative reverse transcriptase–mediated polymerase chain reactions (RT-PCR) were performed in a one-step way using Qiagen One-Step RT-PCR Kit. Oligonucleotides used for RT-PCR reactions are listed. The cycling conditions were chosen according to the protocol and the number of cycles was chosen after testing, according to the number of copies of the cDNA to just make it visible on the gel. After amplification, the RT-PCR products were loaded on 1% agarose gel containing EtBr, scanned with Typhoon 8600 Phospho imager (Amersham Biosciences), and quantified using the ImageQuant software (Molecular Dynamics, Sunnyval, CA)

Table 2. Listed below are primers used for RT-PCR.

Gene of interest	RT-PCR primer pair	PCR product size (gDNA) in kb	PCR product size (cDNA) in kb
ACTINE2	actine2-a + -b	0,44	0,35
RAN3	ran3-a + -b	1,3	0,53
ECR	Ecr-RT-a + -b	0,67	0,57
FAH1	fah1-RT-a + -b	0,6	0,36
FAH1-homol.	fah1-HOM-RT-a + -b	0,72	0,4

2.10. Isolation of DNA fragments from agarose gel

The “QIAquick Gel Extraction Kit” by Qiagen (Hilden) was the kit of choice to isolate DNA fragments from agarose gels.

2.11. Primers

Oligonucleotides were synthesized by Invitrogen (Karlsruhe), Operon (Köln) and Sigma (Hamburg).

2.12. Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify specific DNA fragments on a Biozym MultiCycler Peltier PTC 225. Either Taq polymerase, the “Expand High Fidelity PCR System” or the “Expand Long PCR System” from Roche Biochemicals (Mannheim) was used. Reactions were performed as indicated by the company.

2.13. DNA sequencing and sequence analysis

DNA sequences were determined by the MPIZ DNA core facility (ADIS = Automatische DNA-Isolations- und Sequenziereinheit) on Applied Biosystems (Weiterstadt, Germany) ABI Prism 377 and 3700 sequencers using BigDye terminator chemistry. Premixed reagents were from Applied Biosystems. DNA and protein sequence analysis was performed using the MacVector program (Oxford Molecular Group). Sequences determined from the PCR products were "BLASTed" against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) and after identification annotated according to the MIPS (http://mips.gsf.de/proj/thal/db/search/search_frame.html) and TIGR Arabidopsis sequence databases (http://www.tigr .org/tdb/e2k1/ath1/LocusNameSearch.shtml).

2.14. Map based cloning

2.14.1. Generation of recombinants

I generated recombinant lines by crossing the mutant *cer10* and *cer13* (both on the *L.erecta* background) to the Columbia WT or the other way around. Approximately 100 crosses were carried out and the adult F2 generation used for amplification and mapping. F2-lines exhibiting the mutant phenotype were carefully chosen for the mapping procedure.

2.14.2. DNA isolation in 96 well plates

The DNA isolated as described by Michaels and Amasino (2001) in a 96-well format using a tissue lyser and a subsequent precipitation step.

2.14.3. Databases for SNP

There were two databases available that provide single nucleotide polymorphisms between *L. erecta* and Columbia. These were allocated by the company Monsanto (https://www.arabidopsis.org/cgi-bin/cereon/cereon_login.pl) and the MASC SNP DB from the Max Planck Institute of Plant Breeding Research in Köln. (http://www2.mpiz-koeln.mpg.de/masc/).

2.14.4. PCR for map based cloning

Primers were designed around the SNPs with a size of 150 to 350 bp. A Taq polymerase was used to amplify the fragments around the SNPs. A standard PCR program was used: 95°C for 1 min (denaturation), 95°C for 30 sec (denaturation), 57°C for 1min (annealing of primers), 72°C for 1 min 50 sec (ampification), 35 cycles the last 3 steps, then 72°C for 5 min for final amplification and 15°C for ever.

2.14.5. Separation with denaturing HPLC

The analyses were carried out on a Transgenomic dHPLC WAVE DNA Fragment Analysis system equipped with a DNASep column (Transgenomic). The mobile phase was 0,1 M triethylammonium acetate (Transgenomic), pH 7, and 0,1 mM Na_4EDTA (Transgenomic). DNA fragments were eluted at a flow rate of 0,9 ml/min in a gradient with acetonitrile (Sigma). The predicted melting profile of the provided standard was firstly analysed by the supplied WAVEMAKER (Transgenomic) software to check the correctness of the temperatures and gradient conditions of the machine. For the calibration step, the PCR reactions from heterozygous and homozygous lines were directly loaded on the dHPLC and we determined the optimal temperatures, injecting 5 µl per run. In a successful calibration the chromatogram of a heterozygous fragment differed from the homozygous fragments. For SNP mapping, amplicons derived from recombinants, homo- and heterozygous control lines were run at the established conditions (Fig.3).

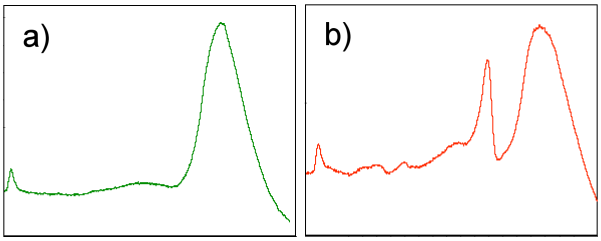

Fig.3. Examples of seperation of PCR fragments on dHPLC at optimal temperature.
a) Seperation of fragment homozygous for SNP
b) Seperation of fragment heterozygous for SNP

2.14.6. Analysis of data

Chromatograms were examined in the WAVE software (Transgenomic) visually for differences between the homoduplexes of Columbia or *L. erecta* only and the Columbia/*L. erecta* heteroduplexes. Only markers with clearly resolved homoduplex and heteroduplex species were used for subsequent genotyping. The map of the region was drawn in Excel. All information coming from the HPLC analyses about heterozygous and homozygous lines were plotted on an Excel diagram in the correct order of the SNPS on the chromosome in the region of interest.

2.15. Biochemical analyses of lipids

2.15.1. Wax analysis

For the wax analysis of *A. thaliana* leaves, 10 rosette leaves of different five-week-old plants were cut and directly dipped in a vial containing pure chloroform for 10 s at room temperature and scanned for area measurement.

For the analysis of waxes of *O.sativa* leaves, the first leaf of 3 plants were taken for one sample, and 4 samples were prepared. Big glass test tubes were rinsed with chloroform for cleaning and the 3 leaves per sample were extracted for 10 seconds in the test tube filled with chloroform. Another test tube was prepared and preheated to 35°C and the rice leaves were extracted a second time immediately after the first extraction. Then the leaves were scanned to calculate the surface area later. The remains of the leaves were cut into small pieces and extracted in chloroform/MeOH to remove soluble lipids prior to the analysis the cutin of the rice leaves as described later.

To the chloroform extracts of the Arabidopsis and the rice wax, 10 µg of tetracosane (Fluka) was added as an internal standard. The surface area of the dipped leaves was calculated with Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA) after the leaves were scanned. After the extraction of the leaves, the solvent was evaporated under a constant nitrogen stream and BSTFA (bis-(*N*,*N*-trimethylsilyl)-trifluoroacetamide (Machery-Nagel, Düren, Germany) was added in pyridine for 40 min at 70°C to convert free hydroxyl and carboxyl groups into their TMS-esters or –ethers. After that the samples were transferred into a GC-vial and run on GC FID and GC-MS. The wax composition was identified by their EI-MS spectra (70eV, *m/z* 50-700) after capillary GC (DB-1, 30 m x 0.32 mm, 0.1 µm (J&W) on an Agilent 6890N gas chromatograph combined with a quadrupole mass-selective detector 5973N (Agilent Technologies, Böblingen, Germany). Injection occured at a column temperature of 50°C, than the temperature was held at 50°C for 2 min, then the wax constituents started to get off the column when the temperature was raised according to the profile: 40°C min^{-1} to 200°C, 2 min at 200°C, 3°C min^{-1} to 310°C, 30 min at 310°C. The flow rate of the Helium carrier gas was 2 ml min^{-1}. For the quantitative determination of wax components the samples were run on a GC equipped with a flame ionization detector.

2.15.2. Bound Lipid Analysis

Prior to the bound lipid analysis the leaves to be extracted were scanned to determine the surface area using the software Adobe Photoshop. After scanning the approximately 30 total leaves were extracted in MeOH/chloroform (1:1, v:v) and the solvent for extraction was changed daily for 14 days. After the last day the leaves were dried under the hood and taken for analysis.

2.15.3. Chemical degradation of polyesters

Two methods for the degradation of the bound lipids were applied to the extracted *A. thaliana* leaves to compare their efficiencies of the analyses of cutin monomers. Firstly transesterification was carried out using borontrifluorid in methanol (BF_3/MeOH) a releasing solvent to extract methylesters for the GC and GC/MS analysis. Secondly the polyester was transesterified by methanolic-HCl (MeOH/HCl) in preparation to the analysis on GC and GC/MS.

2.15.4. Depolymerisation with methanolic-HCl

30 to 50 mg of totally extracted leaves were transesterified by incubating them in 6 ml 1N MeOH/HCl (Supelco) for 2 h at 80°C. After the incubation, 20 µg internal standard (Dotriacontane) was added and the hydrophobic monomers were 3 times extracted in hexane. The extracts were combined and evaporated under a stream of nitrogen. The conversion of the free hydroxyl and carboxyl groups into their trimethylsilyl (TMS) ethers and esters was conducted by adding bis-(N, N-trimethylsilyl)-trifluoroacetamide (Machery-Nagel, Düren, Germany) in pyridine for 40 min at a temperature of 70°C prior to GC-MS or GC-FID analysis.

2.15.5. Depolymerisation with borontrifluorid in methanol

Extracted leaves were transesterified by adding 1 ml 10 % BF_3 in MeOH and incubating at 70°C for 16 hours. This is a very common extraction method to obtain most fatty acid derivatives as their methyl esters. Anhydrous methanol was added in large excess and boron trifluoride acted as a catalyst. After the transesterification, 50 µl of the internal standard (Dotriacontane) was added to the sample. In a new vial, the sample was transferred onto 2 ml of $NaHCO_3$ which caused the mix to develop gas and a bit of foam. This mixture was extracted three times by adding 1 ml of chloroform, vortexing and transferring the lower chloroform phase into a new vial. The united chloroform phases were washed two times with water to get rid of all hydrophilic compounds. The samples were than dried over anhydrous Na_2SO_4. Under a stream of nitrogen the sample volumes were reduced and than transferred into “reacti” vials. All samples were derivatised with bis-(N, N-trimethylsilyl)-trifluoroacetamide as described earlier.

2.15.6. GC and GC-MS analysis of depolymerisation products

Identification of monomers was performed by their EI-MS spectra (70 eV, m/z 50-70) after the separation on capillary GC (DB-1, 30 m x 0.32 mm, 0.1µm (J&W), on-column-injection at 50°C, oven 2 min at 50°C, $10°C^{-1}$ to 150°C, 1 min at 150°C, 3°C min^{-1} to 310°C, 30 min at 310°C and He carrier gas with 2 ml min^{-1}) on an Agilent 6890 gas chromatograph combined with a quadrupole mass selective detector 5973N (Agilent Technologies, Böblingen, Germany). For the quantitative analysis of

the cutin monomers the samples were additionally run on an identical GC-system with a flame ionization detector based on the internal standard. Number of replicates were generally 6 and results were presented in means +/- standard deviation (SD).

2.15.7. Seed coat analysis

For the analysis of the polymers of mature seeds 10 mg of seeds were ground in liquid nitrogen using an agate mortar and pestle. In contrast to china agate cannot be dissolved by chloroform which is needed to remove the ground seeds out of the mortar into tubes. The ground seeds were extracted in MeOH/chloroform for one week at 48°C, rolled on a roller-shaker and the extraction solvent was changed 5 times or more. Of each sample 6 parallels were prepared and analysed. After the extraction of the soluble lipids the crushed seeds were dried under the hood for 24-48 hrs. The seed coat particles were than hydrolysed, transesterified and analysed in the same way as described in the bound lipid analysis. The seeds were weighted prior to the crushing so that the amount obtained for each component could be divided by the mass of each sample.

2.16. Microscopy imaging

2.16.1. Light Microscopy

For light and fluorescent microscopy, the Leica MZFL-III binocular microscope (Wetzlar, Germany) equipped with a digital camera was used to take pictures of roots, inflorescences, flowers and anthers with the Diskuss (Königswinter) software.

2.16.2. Scanning electron microscopy

For scanning electron microscopy, freshly prepared materials were mounted on copper slight and immediately snap-freezed in liquid nitrogen. After that samples were transferred subsequently to a Zeiss DSM 940 electron microscope (Jena, Germany) equipped with a cryo-chamber (Oxford Instruments). After sublimation of

possible ice on their surfaces, samples were sputter-coated with gold and examined at an accelerating voltage of 5 kV at 15 mm. For cropping and assembling of the images Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA) was used.

3. Results

3.1. Analysis of cutin in *A. thaliana*

Classically cutin was analysed from isolated cuticles (Schönherr and Riederer, 1986). During the preparation of the cuticles the plant material was digested in an enzymatic solution of cellulases and pectinases to desintegrate all cell wall components. Generally, cuticles were thick enough (e.g. ivy, olive, tomatoe) to be clearly visible in the enzymatic solution and could easily be recovered for analysis. After complete digestion, all soluble lipids were extracted with chloroform. The remaining cuticle was transesterified, derivatised and subsequently analysed on GC-FID or GC/MS. However, this method could not easily be applied to Arabidopsis. For Arabidopsis, Franke et al (2005) showed that there is an easier method to analyse cuticle components in which the digestion of the leaves is obsolete. Instead of the digestion and washing of cell wall material in an enzymatic solution the fresh tissue is extracted thoroughly in MeOH/Chloroform and after the transesterification of the extracted material the hydrophilic cell wall components are removed by extraction in hydrophilic and – phobic solutions. The lipophilic components are then derivatised and can be analysed. This method resembles total polymeric lipid analysis of the leaf but Franke et al (2005) could show that the amounts of cutin components in isolated and digested cuticles and the analysis of polymeric lipids are similar.

To optimise the transesterification and extraction for the preparation of the cutin components the depolymerisation was carried out with different chemicals: methanolic hydrochloric acid (MeOH/HCl) and borontrifluorid in methanol (BF_3/MeOH). Subsequently, lipophils were extracted using two organic solutions: hexane or chloroform. For the 15 most prominent peaks in the two analyses the amount of cutin monomers was the same for both methods (Fig.4). Using BF_3/MeOH produced more carbohydrates from the cell wall than the extraction in chloroform. In comparison to hexane the chloroform is more hydrophilic and more hydrophilic compounds such as carbohydrates are extracted. As both transesterification and extraction methods produced essentially the same amounts of the cutin components the easier method was chosen for subsequent analyses. When the method with

BF_3/MeOH was used the extraction was more laborious than with hexane because the chloroform fraction has a higher density than the hydrophilic fraction and could not be absorbed from the extraction vials as easy as the hexane.

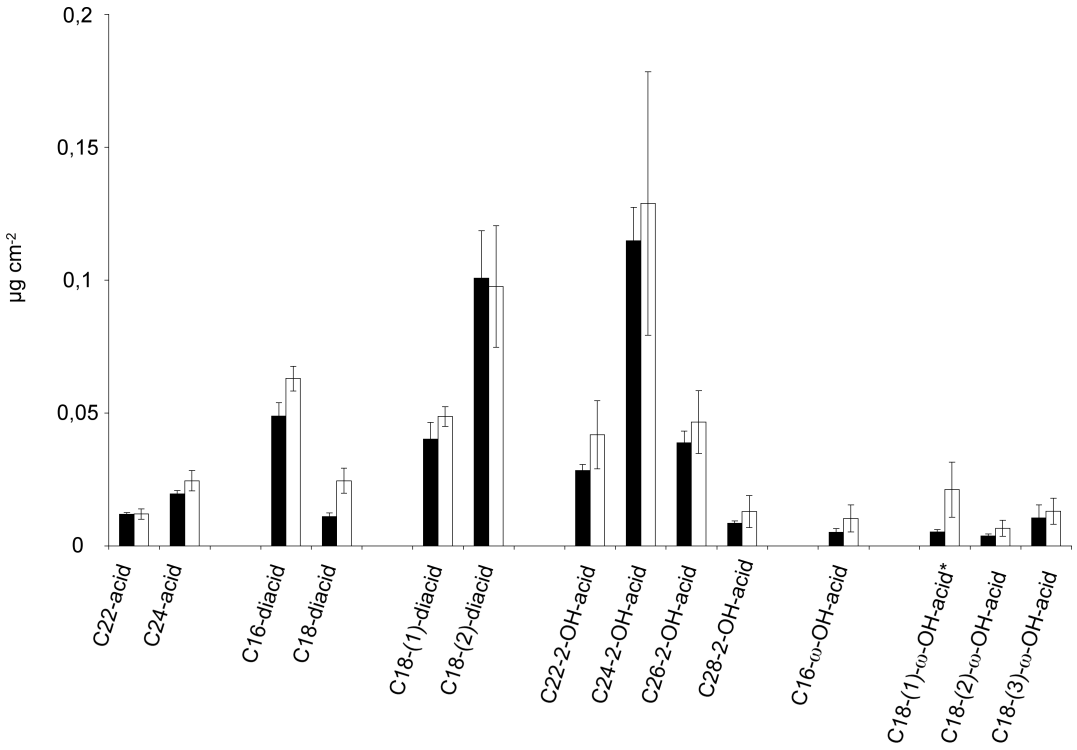

Fig.4. Comparison of methods for cutin isolation.
Description of the methods in the text. The major 15 peaks of both analyses are shown. black: transesterification with hydrochloric methanol and extraction in hexane; white: transesterification with borontrifluorid in methanol and extraction in chloroform. * indicates C18-(1)-ω-OH-acid/C16-9/10-OH-diacid where the second compound lies underneath the other.

3.2. Analysing mutants: Cutin analysis and related experiments

Many cuticular mutants have been described in *A. thaliana*. Among them are *fiddlehead* (Yephremov et al., 1999, Lolle et al., 1992), lacerata (Wellesen et al., 2001), *wax2* (Jenks et al., 2003), *ace/hothead* (Jenks et al., 2004). Of the several Arabidopsis *eceriferum* mutants lacking epicuticular waxes (Hannoufa et al., 1993; McNevin et al., 1993; Jenks et al., 1995, 1996a), only cer10 and cer13 have been reported to show slight adhesion or fusion of organs in the shoot. For gaining a deeper understanding of the function of these genes the cutin analysis was carried out on known and putative cuticular mutants.

3.2.1. Cutin and wax analysis of *ace*

In our lab the APB24 Arabidopsis En/Spm transposon mutant was detected in a collection of lines due to its variations in siliques and inflorescences (Kurdyukov et al., 2006). Prior to flower development mutant plants were indistinguishable from wildtype whereas the APB24 calyces were not able to fully unfold and occasional fusions between buds occurred at a later stage. SEM analysis of petals revealed a marked cell-morphology phenotype with some somatic reversions in cells to the *ACE/HTH* allele (Kurdyukov et al., 2006). For example these cells could be identified between the mutated conical cells on petals. It was shown that APB24 was allelic to *ADHESION OF CALYX EDGES* and *HOTHEAD*. Inflorescences were analysed by TEM and especially the cuticles were examined, as defects in cuticles are believed to cause organ fusions. TEM images of *ace/hth* revealed that the cuticles are sometimes discontinuous and in some spots multilayered. In zones with organ fusions the cell walls were lacking the cuticular covering and the cell walls of the epidermal cells touched each other (Kurdyukov et al., 2006). As *ace/hth* is a putative alcohol dehydrogenase according to sequence similarities and could be involved in the biosynthesis of cutin components according to its phenotypes, the mutant was analysed biochemically. Lipid profiling was conducted comparing all soluble lipids of *ace/hth* to the wild type from the same segregating families. The lipids were analysed by GC-MS and a difference in the amount of five lipids was found. A remarkable increase was detected in the 18-hydroxyoctadecadenoic acid and reduced were ω-oxo fatty acids of the chain lengths C16 and 18. Furthermore the α-ω-diacids with the chain length of C16 and 18 were also reduced. The α-ω-diacids might be the product of the oxidation of ω-oxo-acids. And α-ω-diacids were part of the Arabidopsis cutin as shown by the comparison of methods and Franke et al (2005). As *ACE/HTH* shows homology to oxidoreductases there is a possibility that it functions as a dehydrogenase in the cutin biosynthesis.

The analysis of cutin was carried out according to the method chosen for cuticle analysis for *A. thaliana* with leaves from *ace/hth* and the results were compared to wild type leaves. This cutin analysis revealed that most cuticular components are the same in the mutant and the wild type but that there are some major changes (Fig.5). In the *ace/hth* cuticle there are less α-ω-dicarboxilic fatty acids

(C16: 89,8 % and C18: 83,0 %). The amount of these precursors of the fatty acids the ω-OH-fatty acids is remarkably increased (C16: 168,0 % and C18: 128,0 %). The differences in those components might have even been higher if the mutants did not have somatic reversions to the *ACE/HTH* allele in some cells.

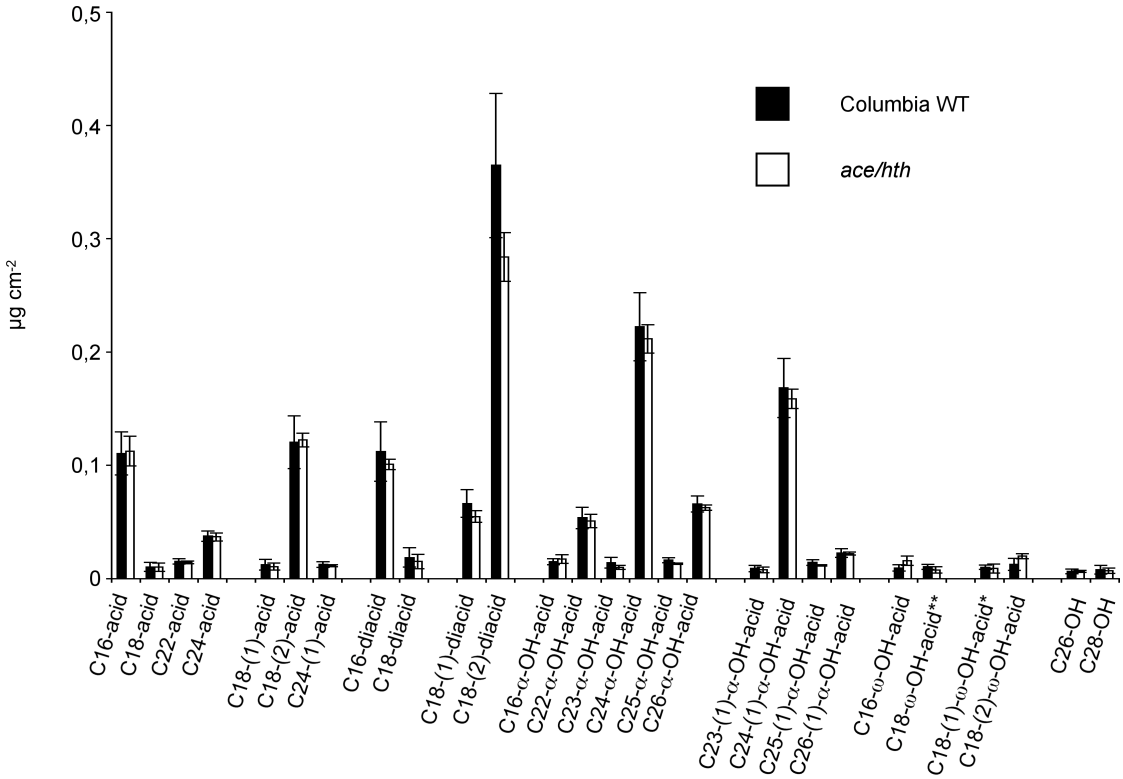

Fig.5. Comparison between the cutin composition in Columbia and *ace/hth* leaves. * indicates C18-(1)-w-OH-acid/C16-9/10-OH-diacid where second compound lies underneath. ** indicates C18-w-OH-acid/C16-10/16-di-OH-acid where second compound lies underneath.

As some fusion mutants exhibit a wax phenotype besides the cuticle phenotype (e.g. *cer10, cer13, wax2*) and due to the fact that the biosynthetic pathways of waxes and cutin components have some overlaps the waxes of *ace/hth* were also analysed (Fig.6). Waxes of rosette leaves were extracted by drowning 10 leaves from one plant for 10 s into chloroform. The volume of these wax extracts was then reduced under a stream of nitrogen and the waxes were then derivatised with BSTFA. The compounds could be identified by GC-MS and quantified by GC-FID. The wax analysis was carried out two times and showed that *ace/hth* has no major changes in the wax amount and composition (Fig.6). That might tell us that *ace/hth* is not involved in the production and transport of the components needed in the wax

biosynthesis. It also means that the lack of ACE/HTH does not lead to a compensatory effect in the biosynthesis of the cuticle components or waxes in leaves. At least not to the extent that it could be detected as an unspecific increase of compounds in the analyses carried out.

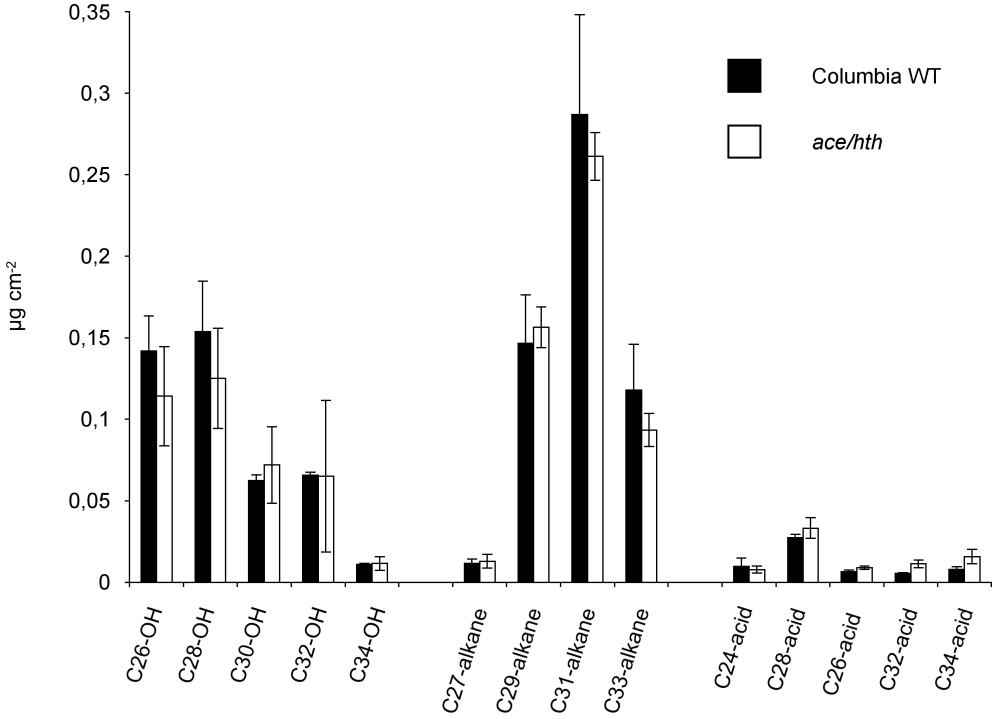

Fig.6. Wax load of Columbia wild type and *ace/hth*.

Krolikowski et al (2003) proposed that *ACE/HTH* is expressed ubiquitously throughout the plant. However, it could recently be shown that *ACE/HTH* is specifically expressed in the epidermis (Kurdyukov et al., 2006). This supports our findings that *ACE/HTH* is involved in the cuticle biosynthesis and more particularly in the oxidation of ω-OH-fatty acids into ω-oxo-fatty acids that was proven by the cuticle analysis.

3.2.2. Cutin analysis of *bdg*

Cutin analysis was carried out on totally extracted *bdg* and wild type leaves for which plants were grown under the same conditions. As in all other cutin analyses the amount of each monomer was quantified as µg per cm^{-2} as the leave surface area was estimated after the scanning of each leave prior to the exhaustive extraction of the leaves in MeOH/chloroform. The amount of each compound could than be

calculated by the means of the internal standard (100 µg of C32-alkane). In the *bdg* mutants, leaves could not be measured by scanning the leaves, as most leaves on these plants were fused and could not be flattened. So the leaf surface area of *bdg* was calculated by known ratios of leaf surface and dry weight. To avoid mistakes due to differences in this ratio between *bdg* and wt, the ratio for leaf surface to dry weight for intact *bdg* leaves was taken into account for the calculation of the leaf surface of strongly fused *bdg* leaves. All components identified by this method in previous experiments were found in the *bdg* mutant as well as in the wild type and, strikingly, the amount of all monomers was elevated in the mutant 1.2 to 3 times (Fig.7). This was the case for the more classical cutin components (hydroxylated fatty acids, diacids, etc.) as well as for compounds that are not classical cutin monomers (acids, alcohols, α-OH-acids, etc). There was no specific reduction in one compound or in a compound class or an elevation in a compound but rather an overall accumulation of all bound lipids of the cell wall. The amount of cutin and cell wall lipids was calculated in percent and in this calculation it could be seen that the ratios between the compounds were the same as in the wild type. In the case of *bdg* there is apparently no defect in a specific pathway but rather an overall upregulation of the synthesis of all cutin and cell wall bound lipid components.

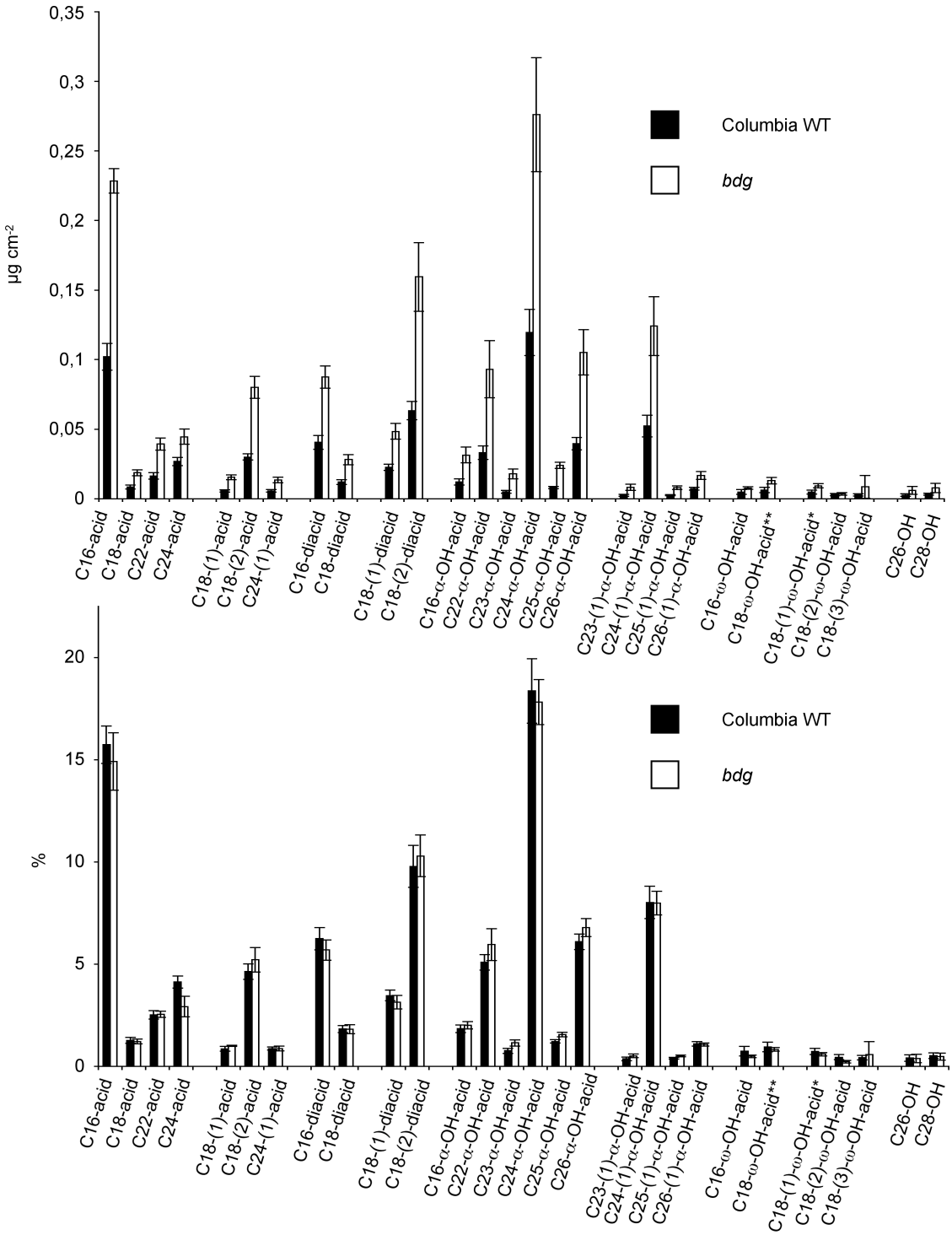

Fig.7. Comparison between cutin components in Columbia wild type and *bdg*. Values are given in µg cm^{-2} in a) and in percent in b). * indicates C18-(1)-w-OH-acid/C16-9/10-OH-diacid where second compound lies underneath. ** indicates C18-w-OH-acid/C16-10/16-di-OH-acid where second compound lies underneath.

The same result was obtained when the amount of wax was measured on *bdg* leaves (Kurdyukov et al., 2006). The wax load accumulates 3.5 times per square centimetre in the mutant compared to the wild type (Kurdyukov et al., 2006). The

increase of the wax components and expression analysis carried out using RT-PCR showed that the decarbonylation pathway is upregulated. The wax biosynthesis products of the decarbonylation pathway are highly elevated (e.g. up to 9.4 times for C29 alkane) and the expression of genes that are known to be involved in the decarbonylation of fatty acids are upregulated up to 184% (*CER1*) and 158 % (*SHN1/WIN1*) (Kurduykov et al., 2006). The function of BDG can not be proposed by its belonging to a protein family (α/β-hydrolases) but it is clear that it is involved in cuticle formation and it is assumed that it acts directly as an extracellular synthase. This is assumed due to two reasons: firstly its compensatory effect on the level of cuticular lipids and waxes as this could happen to compensate for the improper esterification of monomers in the cell wall and the cuticle and secondly due to its extracellular localisation (Kurdyukov et al., 2006).

3.2.3. Cutin analysis of FDH::FAE

Being seed specifically expressed *FAE1* is normally not found in the epidermis of Arabidopsis plants. Millar et al (1998) transformed Arabidopsis plants with the *FAE1* gene under the 35S promoter. The ubiquitous expression of *FAE1* led to a reduction of the wax layer by 50 % and to changes in the thylakoid membranes. The changes of a transgenic plant that expresses *FAE1* specifically in the epidermis, were investigated. After the cloning of *FDH* (Yephremov et al., 1999) a transgenic Arabidopsis line was created that expressed the fatty acid elongase1 (*FAE1*) under the *FDH* promoter (S. Kurdyukov, personal communication). The FDH::FAE mutant exhibited no fusions but a trichome phenotype, no trichomes were visible on FDH::FAE leaves. Cutin analysis was carried on FDH::FAE plants and on the corresponding wild type. The analysis revealed a change in the cutin composition of FDH::FAE (Fig.8). When the cutin components were grouped according to their functional groups the diacids, ω-OH-acids and alcohols showed the same amounts as in the wild type and the amount of acids and α-OH-acids are increased by 22.3 % and 31.9 % respectively (Fig.9).

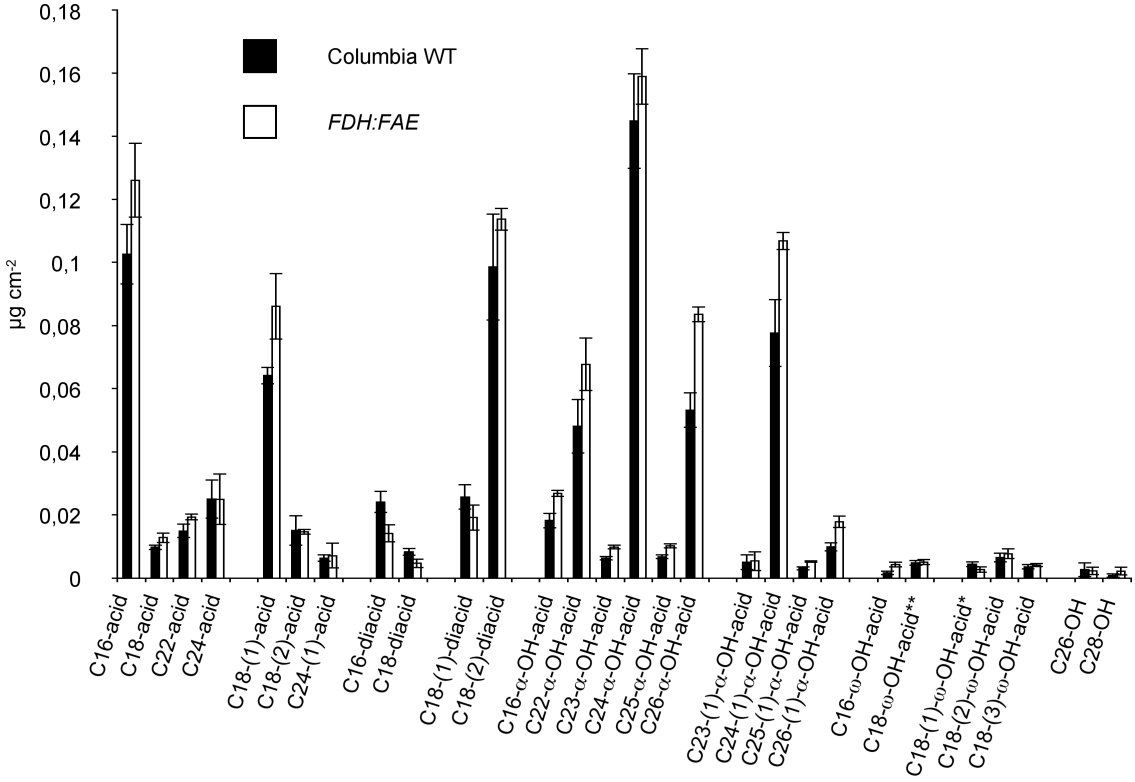

Fig.8.Comparison of cutin composition in the Columbia wild type and FDH::FAE transgenic plants. * indicates C18-(1)-ω-OH-acid/C16-9/10-OH-diacid where second compound lies underneath.
**** indicates C18-ω-OH-acid/C16-10/16-di-OH-acid where second compound lies underneath.**

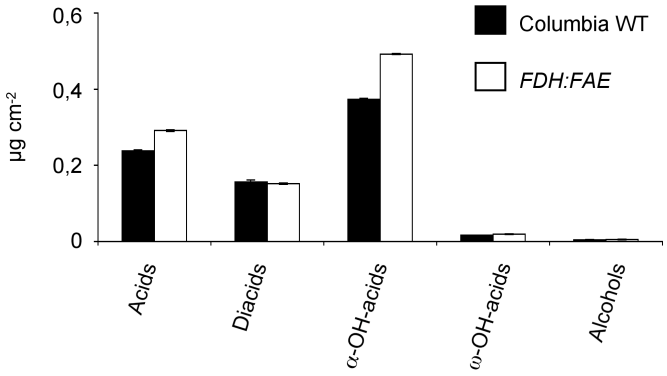

Fig.9.Comparison betweeen cutin composition in the FDH::FAE mutant and columbia wild type sorted according to functional groups.

3.2.4. Analysing *pel3*

With its changed permeability of the cuticle and cell wall and occasional fusions in the inflorescence *PEL3* is an interesting candidate in the cutin biosynthesis. The *pel3* gene was molecularly identified by map based cloning by Tanaka et al (unpublished data, personal communication) and it showed homology to transferases.

Cutin of *pel3* leaves was analysed (Fig.10). The cutin composition of mutant leaves was compared to wild type leaves that grew under the same conditions. The analysis revealed an unspecific increase of all components of the cuticle whereas no functional group or chain length was specifically affected (Fig.10). The amount of diacids was increased 43% more then in the wild type, than ω-hydroxy fatty acids (138 % of the wild type) and α-hydrox fatty acids (119 %). VLCFAs were increased by 18 % and long chain alcohols by 9 %.

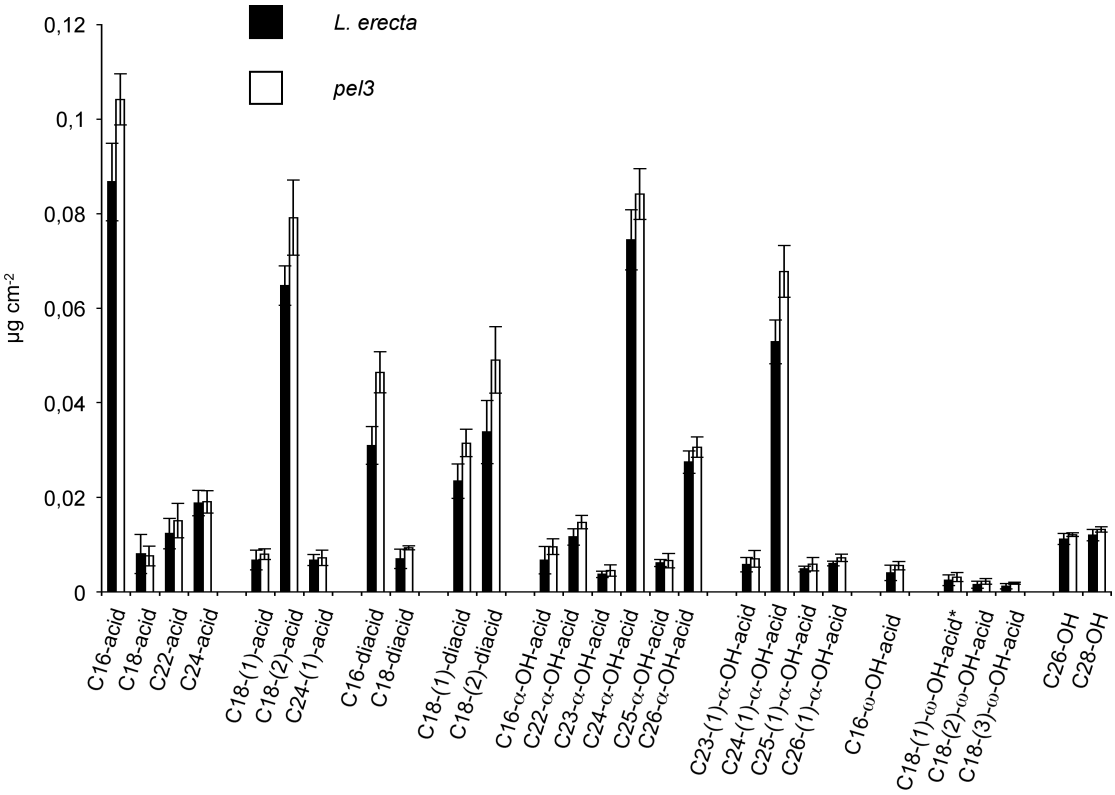

Fig.10. Comparison of the cutin composition in *L.erecta* and *pel3*.
*** indicates C18-(1)-ω-OH-acid/C16-9/10-OH-diacid where the second compound lies underneath the first**

Wax analysis was carried out on *pel3* and the corresponding wild type and the results indicated that *pel3* has no obvious defect in the wax biosynthesis (Fig.11.). The synthesis of cutin components seems to be upregulated but not as much as in *bdg*. In contrast to *bdg*, *pel3* does not show an increase in wax components.

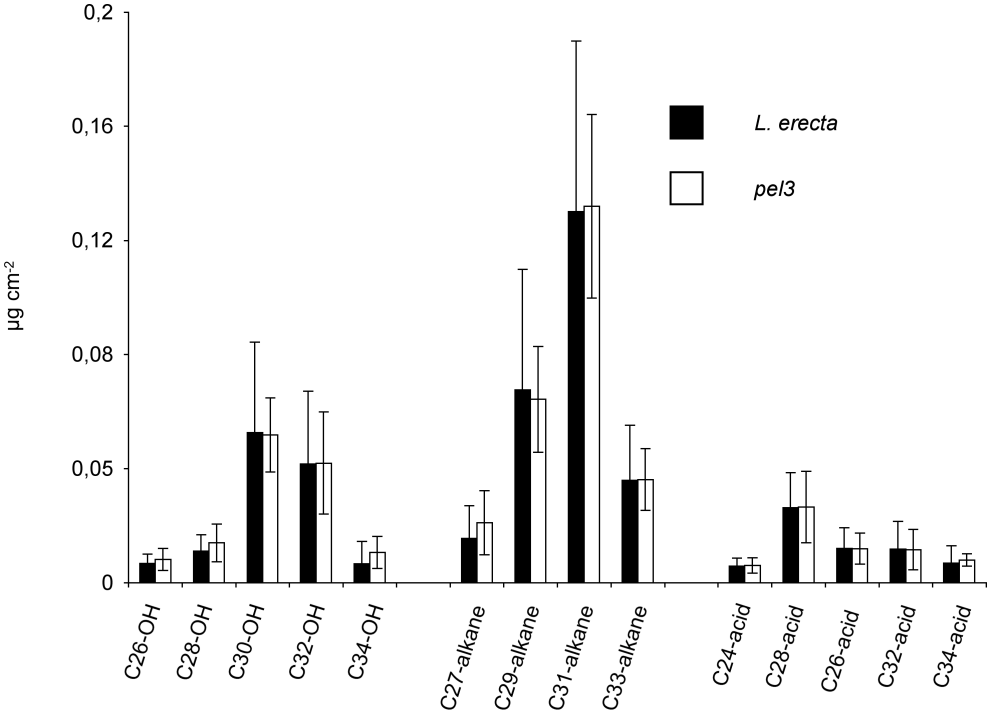

Fig.11. Wax load on leaves of *L.erecta* wild type compared to *pel3* mutant.

SEM analysis of the seeds of *pel3* disclosed an aberrant seed coat (Fig.12). The surface of Arabidopsis seeds seen on SEM pictures is generally the product of the process of seed maturation. During this process the various layers of the seed coat die at different times and in a specific sequence. Mucilage is secreted and deposited during the process and the vacuole contracts to leave a cytoplasmic column in the centre of the cell surrounded by a donut-shaped apoplastic space. Some genes regulating the whole process are known (Haughn and Chaudhury, 2005). The structure of the seed coat resembles the shape of the former epidermis that is preserved by the mucilage and the columella. It is characterized by a flattened columella in the seed coat epidermis and the specific surface pattern. The seed coat phenotype of *pel3* is comparable to the seed coat of *ap2* (Léon-Kloosterziel et al., 1994) as the overall seed shape resembles a heart shape as in *ap2* and has a severe defect in the seed surface. On the surface of the *pel3* seeds the polygonal structure of the epidermal cells is not present in all parts. These seed phenotypes suggest a change in the formation of the epidermal layer, as the columella represents the proper position of the former vacuole. It might also show an involvement of PEL3 in the process of the sequential dying of the epidermal layer of the seed that is a tightly orchestrated process in which unknown players take part (Haughn and Chaudhury, 2005).

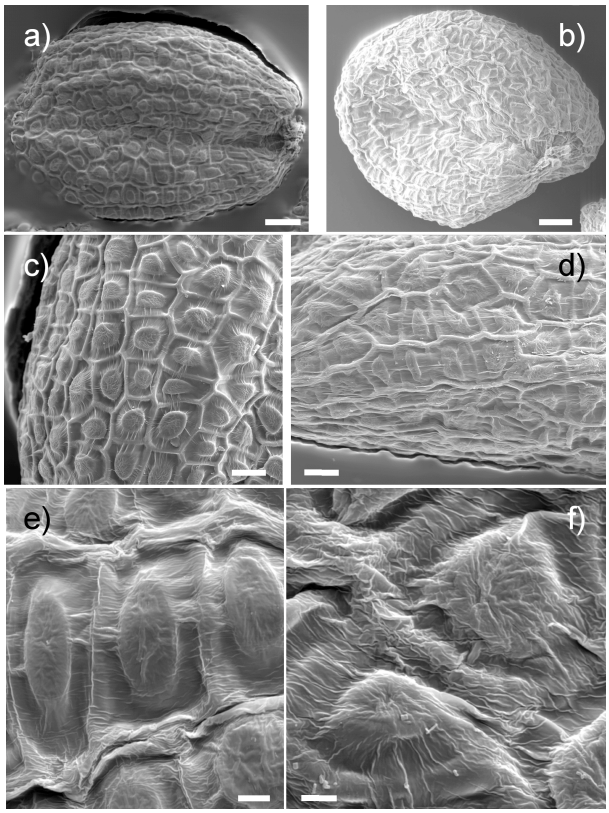

Fig.12. The *pel3* seed coat phenotype
a) *L.erecta* wild type seed
b) *pel3* seed resembles rather a heart shape
c) *L.erecta* wild type seed surface showing the well defined rows of epidermal cells with correct shape of the columellae
d) *pel3* seed surface with rather disorganized rows and not evenly distributed elevations
e) Detail of *L.erecta* wild type row of epidermal cells
f) Detail of *pel3* epidermal cells showing slightly disordered formationof the seed coat
Scale bars represent 50 µm in a) and b), 20 µm in c) and d) and 5 µm in e) and f).

3.3. Analysing *cer10*

3.3.1. Morphological and cuticular defects associated with the loss of the *cer10* function

Cer10 is a mutant with a severe wax phenotype easily visible on the whole plant: especially on siliques, sepals, stems, cauline leaves, rosette leaves and cotyledons (Fig.13.). In a previous publication (Rashotte et al., 2001) the waxes of the stem and the rosette leaves of *cer10* were analysed in addition to the waxes of other cer mutants. In *cer10*, there is a shift of wax components to the longer chain lengths. In leaves this is mainly due to the reduction of C29 alkane and the increase of C30 primary alcohol. On stems a similar pattern with reduced C29 components and

increased C30 primary alcohol levels was observed. The authors proposed a putative function of *cer10* in the conversion of the C30 fatty acid to C30 aldehyde (Rashotte et al., 2001).

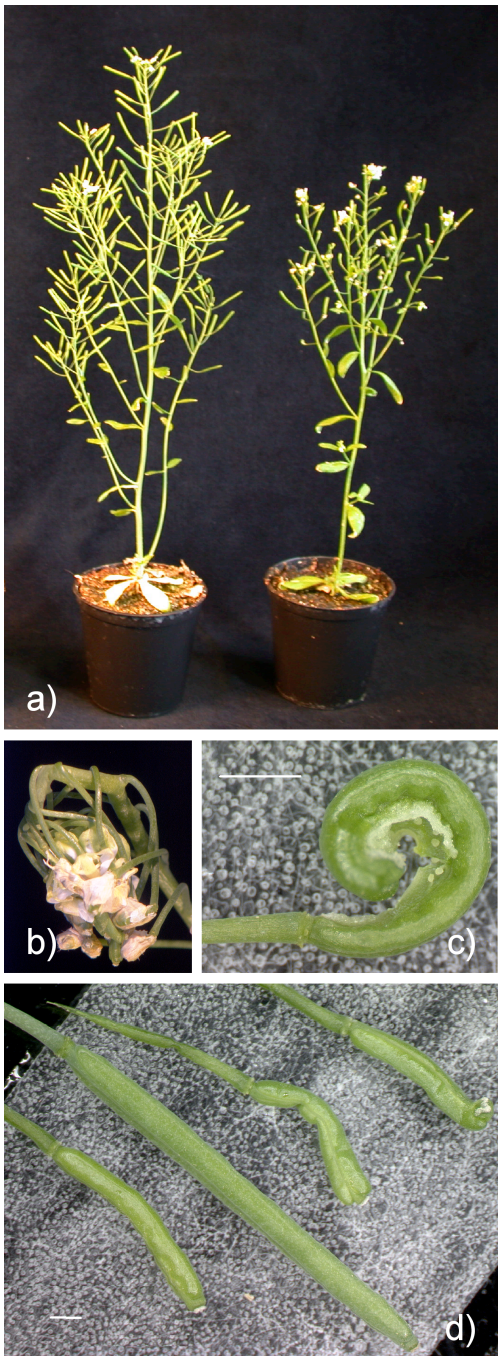

Fig.13. Habitus and silique phenotypes of *cer10*
a) The habitus of wild type Arabidopsis (left) and the *cer10* mutant (right) both in the *L.erecta* genetic background. Apart from the overall glossiness, the *cer10* plants have a stunted growth and a delay in branching. The number of branches is also reduced. b) *cer10* inflorescence shows a *fdh*-like shape c) Siliques of *cer10*: glossy appearance, open at suture, sterile seeds and curved d) The wild type silique is the second from the left, the other three resemble the glossy and slightly curved *cer10* siliques.
Scale bars represent 1 cm.

Besides the wax phenotype, the mutant exhibited further differences when compared to the *L.erecta* wild type. The *cer10* mutant displayed abnormal morphogenesis of all shoot organs (Fig.13.). The first true leaves were crinkled and smaller when compared to the wild type. Overall the mutant had a smaller stature but carried a normal number of leaves without an apparent time delay during development. Siliques were often deformed in *cer10* (Fig.13c). A severe trichome phenotype was visible starting from the first trichomes on developing rosette leaves (Fig.14a) – c). The trichomes seemed to develop normally until they stand up, then they seemed to loose turgor and fell on the leaf until they lay flat on the leaf surface. The support cells at the bottom of the *cer10* trichomes looked differently in comparison to the support cells in *L.erecta* wt. In the *cer10* mutant they were tighter at the bottom of the trichome whereas in the wild type the support cells taken together were wider than the trichome (Fig.14a) – c). The average number of trichome branches was less then three in *cer10* in comparison to the usual three branches usually observed in *L.erecta*. Further differences of *cer10* to the wt were the shape of epidermal cells on cotyledons and on sepals. Apparently, the epidermis cells on those organs were differently organised. On cotyledons, the epidermal cells were smaller and wrinkles occured that were not seen on wild type epidermal cells (data not shown). The sepals of *cer10* showed a different shape in comparison to the wild type and the trichomes on *cer10* sepals were unevenly distributed over the sepal surface and did not fully develop (Fig.14f). Epidermal cells on sepals of *cer10* had unequal shapes and sizes in comparison to the wild type where the epidermal cells on the sepals were all very similar in appearance. Organ fusions in *cer10* were only observed between sepals. When it is the time to open the sepals in *cer10*, to give space for the opening flower the sepals stay in contact and can only be torn apart (Fig.14). That is a possible reason for the flowers to be so underdeveloped and not as fertile as the wt. An additional reason for the reduced fertility could be the reduced number of pollen that can be seen on the stigma of *cer10* in comparison to the wt stigma of the same age. The fusion of the margins of the sepals in *cer10* prevents the flower from opening. The other organs in the flower keep growing and are more and more restricted in their space in the flower. This can be seen when the sepals of the *cer10* flower are removed with forceps and the folded petals, anthers, carpels and the pistil try to unfold. The flowers of the *cer10* mutant still developed seeds even under

these stringent conditions. Another phenomenon that could be observed was the occasional development of multiple organs in the *cer10* flower. About 15% of the

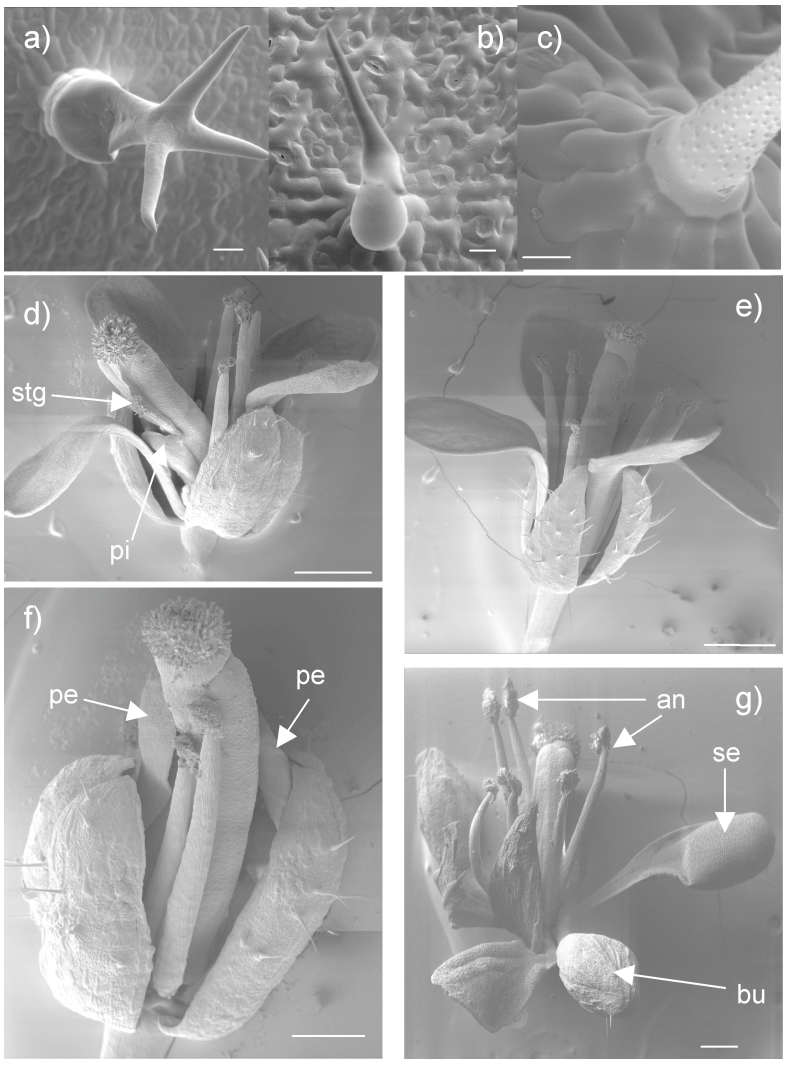

Fig.14. Developmental defects in the *cer10* trichomes and flowers.
a) and b) Trichome of *cer10*
c) Trichome support cells of the wild type
d) Flower of *cer10* showing the fused sepals and the trichome phenotype on sepals. Additionally an extension can be seen which looks like an extra pistil (pi) with stigmatic papillae (stg)
e) Flower of the wild type
f) Flower of *cer10* exhibiting the sepal phenotype and the folded petals (pe). That is because the incorrect opening ot the petals due to the fusion between them
g) Flower of *cer10* with multiple organ: an extra bud is seen emerging from the open bud (bu), seven anthers can be seen in the open bud.
Scale bar represent 20 µm in a)- c), 1 cm in d) and e) and 0,5 cm in f) and g)

plants yielded a flower that carried either an extra flower bud in the flower, an extended number of anthers or petals or an extension of the original pistil or an extra pistil (Fig.14d). In the literature only mutants have been reported in which the phenotype of multiple organs occurs in almost every flower and not as rare as in

cer10 (eg. *wuschel*, *superman*). Likely this phenotype is due to an occasional indirect or direct distortion in signalling due to the disturbed VLFA metabolism. As sphingolipids can serve as important signal molecules in the regulation of plant development, the *cer10* phenotype could be due to direct or indirect effects on sphingolipid biosynthesis or on the sphingolipid pool of the plant membranes (Sperling and Ernst, 2003).

Roots were investigated for their growth behaviour because in other organ fusion mutants a big difference in root growth has been reported (e.g. *bdg*). The roots of the *cer10* mutant appeared to grow normally when checked under tissue culture conditions on vertical plates. One thing was different when the roots were checked under these conditions: the *cer10* plants were repeatedly infected by green algae when the wt never showed any infection even in the same plate.

Under greenhouse conditions, the *cer10* mutants also exhibited a difference to the wild type in terms of infection. The wild type was under specific conditions heavily infected by plant fungi when there was still no sign of a fungal infection on the *cer10* mutant. But these phenomena were not further analysed.

The scanning microscopic analysis was applied on mature seeds of the *cer10* mutants to assess the surface of the seeds. The *cer10* seeds only show minor differences to *L.erecta* wt seeds (Fig.15). The size and shape of the seeds were similar to the wild type. Only the arrangement and the shape of former epidermal cells appeared to be different to the wild type seed coat. The columella and the elevated surroundings of each epidermal cell looked normal in *cer10*.

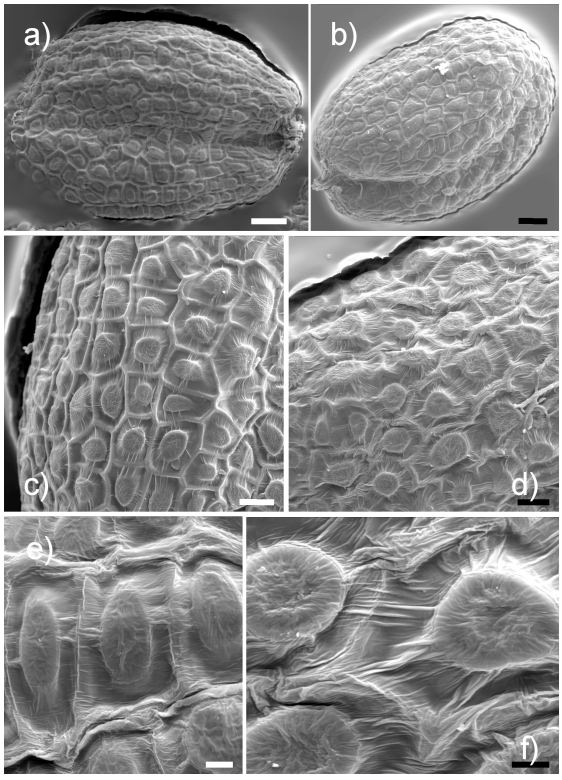

Fig.15. The *cer10* seed coat phenotype.
a) *L. erecta* wild type seed
b) *cer10* seed
c) *L. erecta* wild type seed surface showing the well defined rows of epidermal cells with correct shape of the columellae
d) *cer10* seed surface with rather disorganized rows and not evenly distributed elevations
e) Detail of *L. erecta* wild type row of epidermal cells
f) Detail of a *cer10* row of epidermal cells showing slightly disordered formation of the seed coat
Scale bars represent 50 µm in a) and b), 20 µm in c) and d) and 5 µm in e) and f)

Cloning of the *CER10* gene was tried using a map-based cloning approach. The sequence variation between individuals was detected with SNPs of recombinant lines of cer10 in the *L.erecta* ecotype crossed to Columbia wild type. Denaturing HPLC was used to genotype the polymorphisms in the region where the coarse mapping of cer10 has been reported earlier (Rashotte et al 2004). The result of the mapping was a region spanning three BACs on chromosome 3: T8P19, T29H11and T24C20.

During the process of fine mapping a publication identified a gene as *CER10* in a region 31 BACs or 2,5 Mb away from the previous mapped location. The publication of Zhen et al (2005) described *cer10* as having a mutation in the *ECR* gene At3g55360 with a knock out in the enoyl CoA reductase in the fatty acid synthase (FAS) complex. The original *cer10* line was described as having

duplications and inversions in the promoter region and further than ATG, so by sequencing it could not be proven if *cer10* had a defect in *ECR* as it was not a simple deletion. To prove that the *cer10* line that I worked with was the same as the published one three experiments were carried out: firstly I checked the expression of the *ECR* gene in the leaves of the original *cer10* lines. That revealed a clear reduction of the expression of *ECR* in the original *cer10* line (Fig.16.).

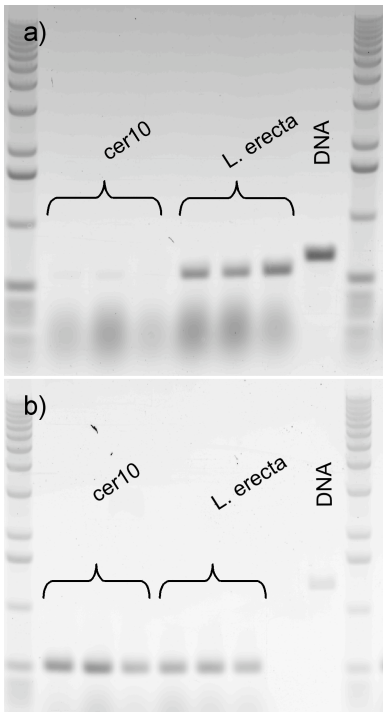

Fig.16. RT-PCR expression analysis of *ECR* in the *cer10* mutant.
a) Expression of *ECR* in *cer10* and wild type plants (product sizes: 570bp for cDNA and 671 bp for genomic DNA)
b) Expression of the *RAN3* control gene in *cer10* and wild type (product sizes: 531 bp for cDNA and 1300 bp for genomic DNA)

Secondly the original *cer10* line was crossed to the homozygous insertion line from salk with the insertion in the At3g55360 gene. This insertion line clearly showed the *cer10* phenotypes. All crosses exhibited the *cer10* phenotype so no complementation of the phenotype was obtained in F1 by the *CER10* allele. The crosses all exhibited in the F1 the Columbia phenotype as the *L.erecta* phenotype is recessive to Columbia. This was the proof that the crosses worked.

Thirdly the original *cer10* line was complemented with the At3g55360 gene under the 35S promoter. The *cer10* mutant and the *L.erecta* wild type were transformed with that construct. Three *cer10* mutants and four *L.erecta* transgenic plants (F1 generation) were resistant to BASTA selection. All three *cer10* plants

appeared to be wild type, the *L.erecta* transgenics also looked like wild type upon first inspection. This finding demonstrated that full restoration of *CER10* was achieved by the complementation of *cer10* with the *ECR* gene. PCR using primers specific for the vector could prove that the complementation was due to the transformation and not a contamination with wild type seeds.

With the results of these three experiments I could prove that the *cer10* mutant was the same as the one published by Zheng et al in 2005. All mutations affect the *ECR* gene.

3.3.2. Biochemical analysis

Having in mind that *CER10* is allelic to *ECR*, the elongation of fatty acids to chain lengths beyond 16 carbon atoms could be impaired as *ECR* catalyses the last step in the fatty acid elongation at the membrane of the ER. Despite that, the spectrum of chain length of waxes in *cer10* is skewed to longer chain lengths. We analysed the cuticle monomers of *cer10/ecr* to see if the knock out of the gene has a direct or indirect effect on the cutin composition. The analysis was again carried out with extracted leaves of which the remains were subjected to transesterification and derivatisation. All cutin components that were found in the wild type were also found in the *cer10/ecr* mutant but the amounts of different molecule classes was different (Fig.17). The bound lipid analysis in the cer10 mutant in *A. thaliana* revealed approximately the same amounts of diacids (~ 90 % of the wild type) for cer10 and the wild type. The quantity of saturated and unsaturated fatty acids (~105 % of the wild type) and ω-hydroxylated fatty acids (~107 % of the wild type) is increased in the *cer10/ecr* mutant and there was a major decrease in α-hydroxy fatty acids (69 % of the wild type) and long chain alcohols (~ 64 % of the wild type) in the *cer10/ecr* mutant when compared to the wild type (Fig.18).

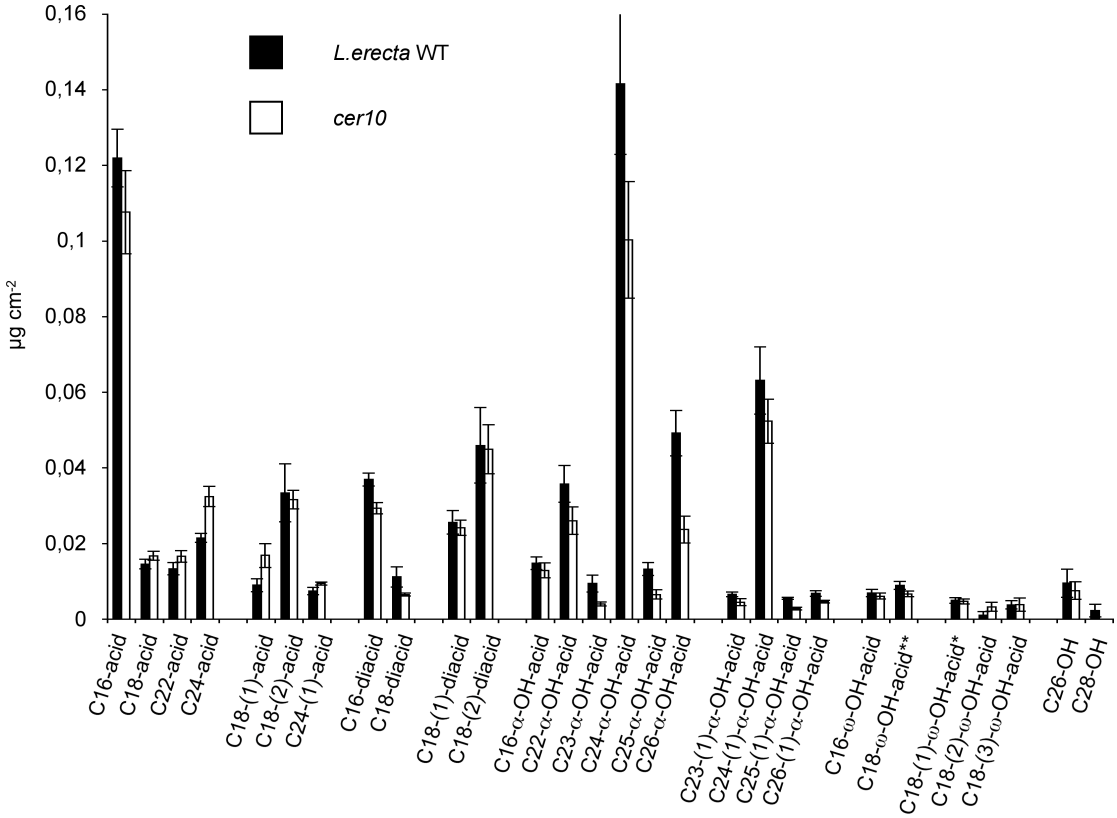

Fig. 17. Comparison of cutin components of *L.erecta* and *cer10*.
*** indicates C18-(1)-ω-OH-acid/C16-9/10-OH-diacid where second compound lies underneath.**
**** indicates C18-ω-OH-acid/C16-10/16-di-OH-acid where second compound lies underneath.**

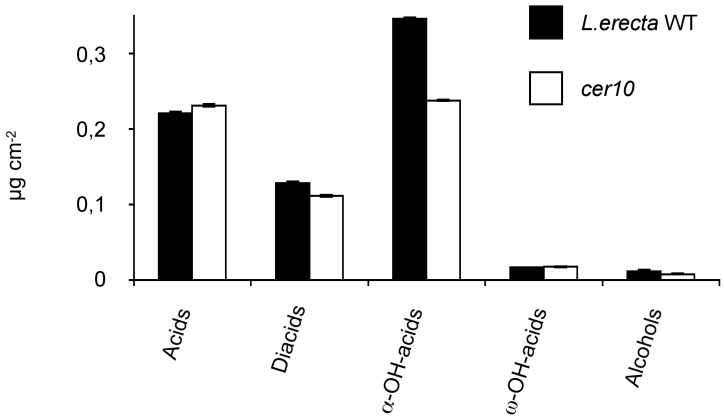

Fig.18. Comparison of cutin components of *cer10* and *L.erecta* sorted by their functional groups.

One explanation might be that when the cuticle advances maturity the plant always tries to compensate if the cuticle is not optimal. For the biochemical analysis of cutin in Arabidopsis only leaves of a specific size can be taken so the plants are

rather old and secondary effects could have already lead to a compensation of a direct effect of a missing protein. An analysis of younger leaves that are not affected by the stresses of an irregular cuticle is not known yet. As a phenotype was seen on seeds we thought that if we find a difference in the polyester composition in seed coat lipids these could be a more direct effect of the mutation. Seeds are in an enclosed space until the seeds are completely dessicated and released from the silique. So the lipids are formed when the organ is not exposed to environmental stresses and the degree of compensation for the function of a missing gene would be lower as on the surface of leaves. For the analysis of seed coat lipids all storage lipids and membrane lipids had to be extracted prior to the transesterification. This was achieved by crushing the seeds with a mortar and pestle in liquid nitrogen. Subsequently the extraction, transesterification, derivatisation and analysis were carried out as described for the Arabidopsis cutin. The seed coat lipid analysis of *cer10* disclosed that a small amount of storage lipids was not extracted prior to the analysis. This varied between the 5 paralleles and yielded a significant standard deviation for two components (C18-(1)- and C18-(2)-acids) (Fig.20.). This improper loss of storage lipids could be due to incomplete seed crushing and storage lipids were still present during the process of transesterification. The same applied for the trans ferulic acid that is part of the membrane in the seeds and underlies the C16-acid and the variation leads to a big standard deviation. The results from the remaining components are clear. When the data are presented grouped according to their chain lengths than one can see that there is a slight accumulation of compounds with 16 carbon atoms, compounds with 18 carbon atoms are 92 % in the mutant compared to the wild type and with the increasing chain length the amounts in the *cer10* mutants become smaller (C20: 78 %, C22: 56 %, C24: 42 %) (Fig.21). The analysis of the bound lipids in the seed revealed a clearer picture of the function of ECR/CER10 which should show an impairment in the elongation of VLCFAs. The effect on the cutin components might rather be a compensatory effect of the plants or an effect due to an improper signalling of lipids that leads to a down regulation of the α-hydroxylation.

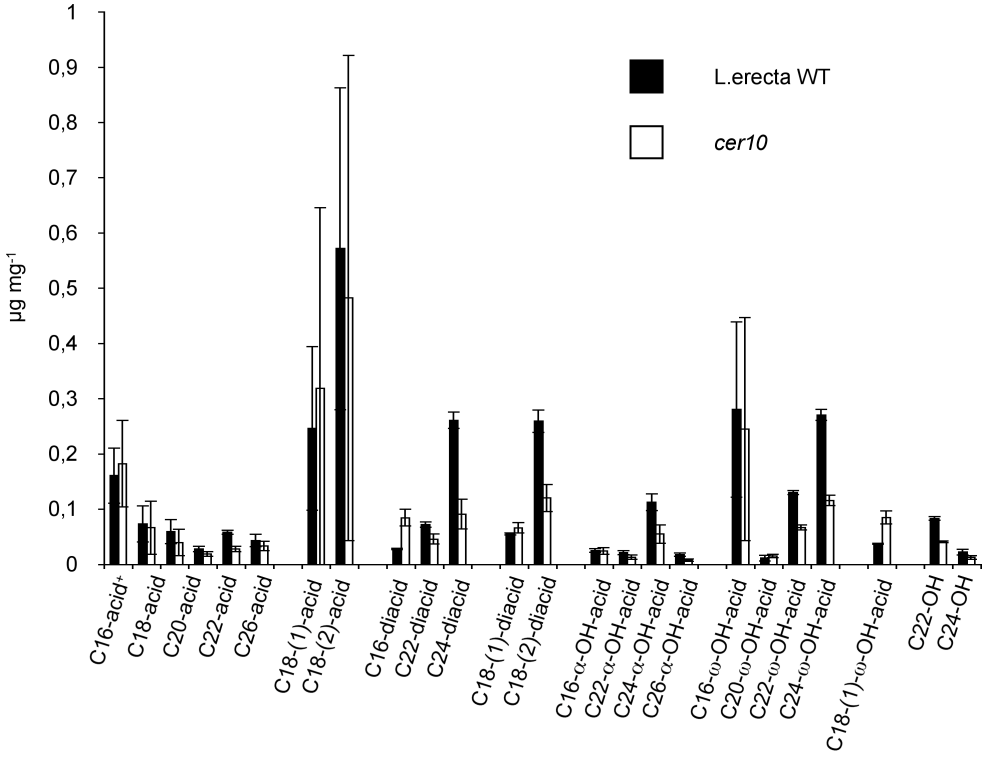

Fig.20.Comparison of seed coat polymeric lipids of *L.erecta* and *cer10.*

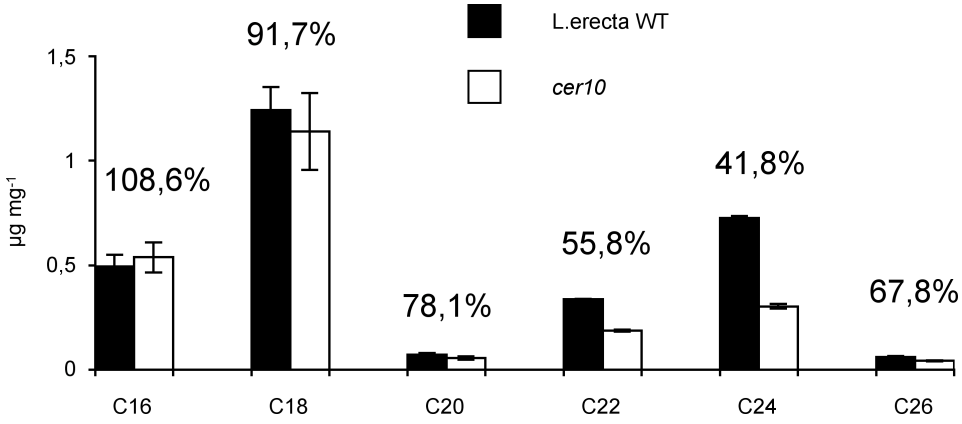

Fig.21.Comparison of seed coat polymeric lipids of *cer10* and *L.erecta*, sorted by their functional groups.

3.3.3. Alpha hydroxylation

It can be assumed that the decrease of α-hydroxy fatty acids in the *cer10/ecr* cutin is due to indirect effects caused by the primary reduction of VLFAs. So far, α-hydroxylated fatty acids have been reported to exist only in sphingolipids (Cahoon and Lynch, 1991; Millar et al., 1998; Bohn et al., 2001; Bonaventure et al., 2003) but not only α-hydroxy fatty acids. Sphingolipids of *cer10* were analysed by Zhen et al (2005). Inositol phosphorylceramides are thought to be the major subclass of

complex sphingolipids (Dunn et al., 2004) but a protocol for purification in Arabidopsis is only available for glucosylceramides (Glc-Cers). Furthermore, the composition of complex sphingolipids in Arabidopsis has not been sufficiently investigated yet. It was shown that *cer10/ecr* has a small reduction in hydroxylated fatty acid content of Glc-Cers compared to the *L.eracta* wild type (Zhen et al., 2005). An analysis reported in the same paper revealed a different amount of all isolated hydroxylated fatty acids demonstrating that there are other hydroxylated fatty acids than the ones in the Glc-Cers. Similar to the hydroxylated fatty acid content in Glc-Cers there is no significant change in all hydroxylated fatty acids but only a slight reduction especially in the ones with longer chain lengths. The author presumed that it is this minimal change that causes the morphological changes of the *cer10* mutant. The argument for the minimal change in sphingolipids to be responsible for the massive change in shape in the *cer10* mutant is that in another experiment the silencing of *cer10* with RNAi constructs in seeds (overexpression with the *FAE1* promoter) or in the epidermis (overexpression with the epidermis specific *CER6* promoter) only resulted in a wax phenotype without the morphological changes. The same phenomenon was detected in other mutants in which only the seed storage lipids were effected or only the wax productin was reduced but in both cases no morphological changes were detected (Kunst et al., 1992; Aarts et al., 1995; Millar et al., 1999).

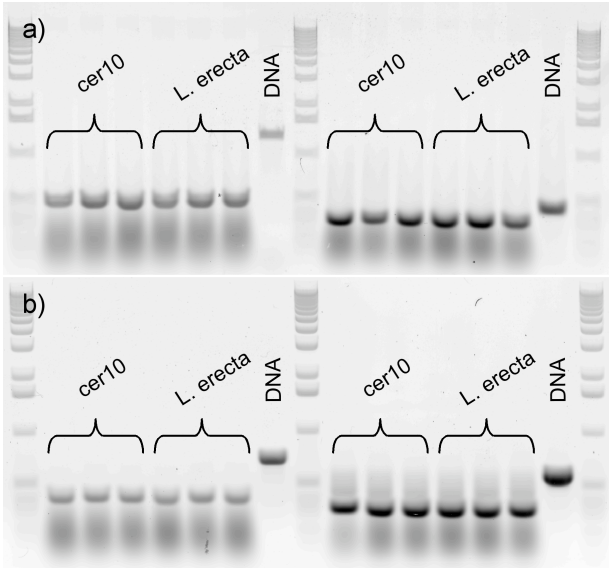

Fig.22. RT-PCR expression analysis of genes putatively involved in α-hydroxylation.
a) Expression of control genes. Left gel: Expression of *RAN3* in *cer10* and the wild type (product sizes for cDNA 531 bp and 1300 bp for genomic DNA). Right gel: Expression of *ACTINE2* (product sizes for cDNA 353 bp and 440 bp for genomic DNA)
b) Expression of genes putatively involved in α-hydroxylation. Left gel: Expression of *FAH1*-homologue (product sizes for cDNA 396 bp and 716 bp for genomic DNA). Right gel: Expression of *FAH1* (product sizes for cDNA 362 bp and 597 bp for genomic DNA)

We expected that the lack of *cer10/ecr* reduced indirectly the production of α-hydroxy fatty acids. Enzymes that could be responsible for this reaction were inferred from the literature. *FAH1* (At2G34770) and its closest homologue *FAH1-like* (At4G20870) were chosen as candidate enzymes being capable of α-hydroxylation. These genes were investigated using a semi-quantitative RT-PCR to assess a difference of expression of these two genes in the *cer10* mutant and the *L.erecta* wild type. For both genes the primers were designed to amplify a 362 bp and 396 bp fragments from RNA and 597 bp and 716 bp fragments from genomic DNA, respectively. The results were standardised to the constitutively expressed *RAN3* or *ACTINE2* gene expression level. The expression of *FAH1* in *cer10* was identical to the WT: 102 % when standardised to *RAN3* and 110 % when standardised to *ACTINE2* and the same was true for *FAH1*-hom: 104 % standardised with *RAN3* and 109 % when standardised with *ACTINE2* (Fig.22).

Therefore, the hypothesis that the two genes *FAH1* and *FAH1-hom* could be responsible for the reduction of α-hydroxy fatty acids in the *cer10* mutant could not be confirmed by the RT-PCR analysis.

3.4. Analysing *cer13*

3.4.1. Morphological and cuticular defects associated with the loss of the *cer13* function

Besides the wax phenotype *cer13* exhibits a striking change in morphology. The growth of the *cer13* plants was stunted, i.e. plants were smaller but they were also more bushy (Fig.23) in comparison to the corresponding *L. erecta* wildtype. Under greenhouse conditions *cer13* never exhibited an organ fusion phenotype which was reported for that mutant in Jenks et al (1996). When *cer13* plants were grown in culture conditions there were also no fusions visible. Roots were investigated for changes in comparison to the wild type and no obvious changes were detected (data not shown).

Fig.23. View of morphology of *cer13* (left) and wild type (right) showing the stunted growth of *cer13* with normal number of shoots and leaves. Both plants are in the *L.erecta* background.

Scanning electron microscopy revealed not visible changes of *cer13* cotyledons, inflorescences, stem and leaves in the organisation of the epidermis on leaves, stem, sepals or petal. Pavement cells on these organs had the same size in *cer13* and the *L.erecta* wild type. The trichomes looked the same as in the wild type and had the same shape and size. Also, *cer13* had no detectable phenotype with regards to the formation of the flower. Seeds of *cer13* were also analysed by SEM and here a change in morphology was visible (Fig.24.). As in *pel3* seeds the phenotype of the *cer13* seed coat surface resembled that of *ap2*. The columella which resembles the former vacuole of the epidermal cells was mostly absent in *cer13* seeds. The pattern of the epidermal cells was visible but not as clear as in the wild type. Apparently the former epidermis was already not in proper shape and when it died during the process of seed development the cytoplasmic column could not be built. The mucilage content of the seeds was not further analysed.

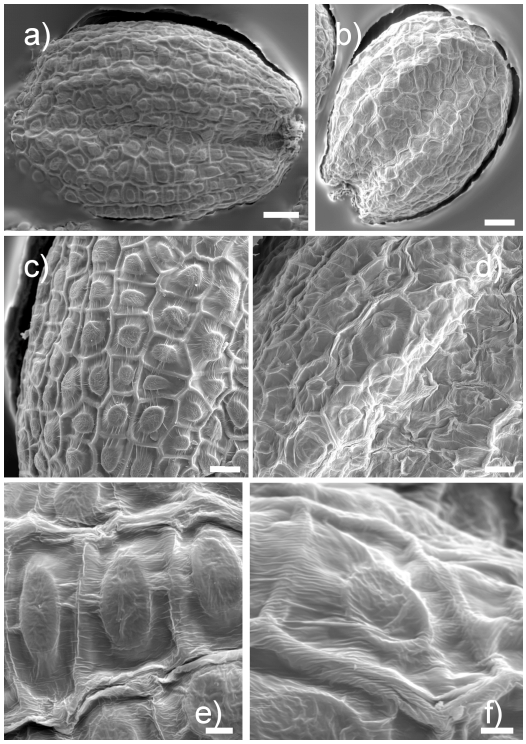

Fig.24. The *cer13* seed coat phenotype.
a) *L.erecta* wild type seed
b) *cer13* seed which is slightly smaller than the wild type
c) L.erecta wild type seed surface showing the well defined rows of epidermal cells with correct shape of the columellae
d) *cer13* seed surface with disorganized rows and not evenly distributed elevations and rather polygonal than square cells
e) Detail of a *L.erecta* wild type row of epidermal cells
f) Detail of a *cer13* row of epidermal cells showing disordered formation of the seed coat
Scale bars represent 50 µm in a) and b), 20 µm in c) and d) and 5 µm in e) and f)

3.4.2. Mapping

As reported in the literature, the mutation in the original *cer13* was produced by fast neutrons (Koorneef et al., 1989) and it was mentioned to be at 66,2 cM (TAIR) and at 47,3 cM (Rashotte et al., 2004) on the lower arm of chromosome 3. For map based cloning a mapping population was established in crossing the *cer13* mutant from a *L. erecta* background with the Columbia wild type. In the F1 generation all plants were heterozygous for the mutation, and the F2 could be taken for the mapping of the gene by recombination. Primers were designed in the previously mapped region (44 to 67 cM) and around SNPs. With PCR and subsequent analysis of the PCR fragments it could be determined if a recombinant line was heterozygous or homozygous at the position of that particular SNP. With the information about the recombination events in the area on chromosome 3 it should be possible to estimate the position of the mutation of *cer13* as only plants showing the *cer13* phenotype

were chosen for PCR and subsequent analysis of the PCR fragments. In the region of the mutation the PCR products will surely be homozygous because otherwise the lines would have not exhibited the *cer13* phenotype. Unfortunately the analysis was limited due to the fact that in the databases the density of SNPs that was available with SNPs (Monsanto and Masc) between *L.erecta* and Columbia were not sufficient in the area of fine mapping of *cer13*. Thus, a PCR with specific primers was carried out to establish a statistical view of the region. All 454 recombinant lines of which DNA was isolated were tested with 3 primers that worked well so that a statistical map could be established (Fig.25). This map pointed to a mapping region of *CER13* between BAC MUO10 and MTO24. A number of genes in this region were found in microarray data to be expressed in the epidermis of upper stems (Suh et al., 2005) and the corresponding insertion lines were ordered to check for wax or fusion phenotypes.

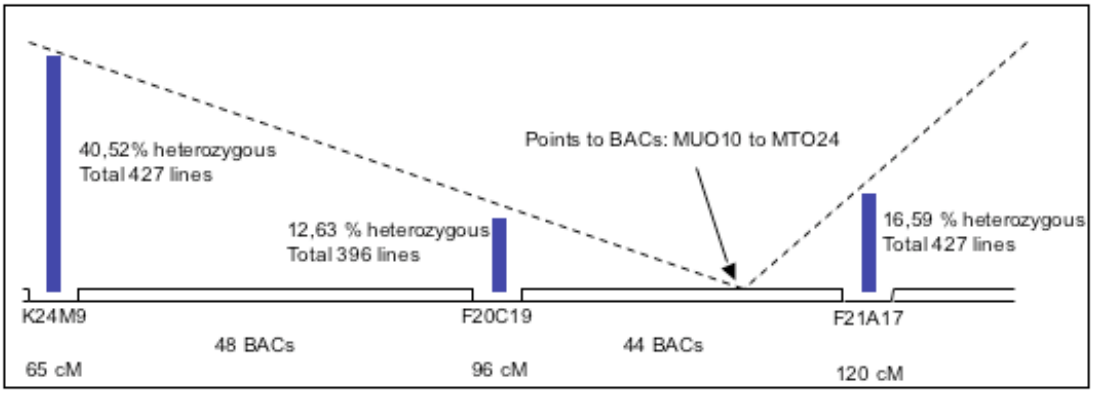

Fig.25. Mapping of *cer13* with 454 recombinant lines using dHPLC.
Schematic representation of the region on chromosome III where *cer13* is located. Blue bars represent amounts of heterozygous *cer13* in SNPs at this position. The dotted line indicates the predicted position of the *cer13* mutation based on the analysis of 454 recombinant lines.

3.4.3. Biochemical analysis of *cer13*

The analysis of cutin components of *cer13* showed some differences when compared to the wild type. Most components were decreased in *cer13* especially the long chain alcohols (63 % of the wild type), the ω-hydroxy fatty acids (75,6 % of the wild type), the α-hydroxy fatty acids and the diacids (both 76 % of the wild type). VLCFAs in *cer13* are 93 % of the wild type (Fig.26). But in all groups the changes are unspecifically distributed among chain lengths. These results resemble an unspecific

change like in some other mutants and could be due to a downregulation of the biosynthetic pathway of cutin and waxes because of the knock out of the *cer13* gene. CER13 might be a transcription factor inducing the biosynthesis of cutin components.

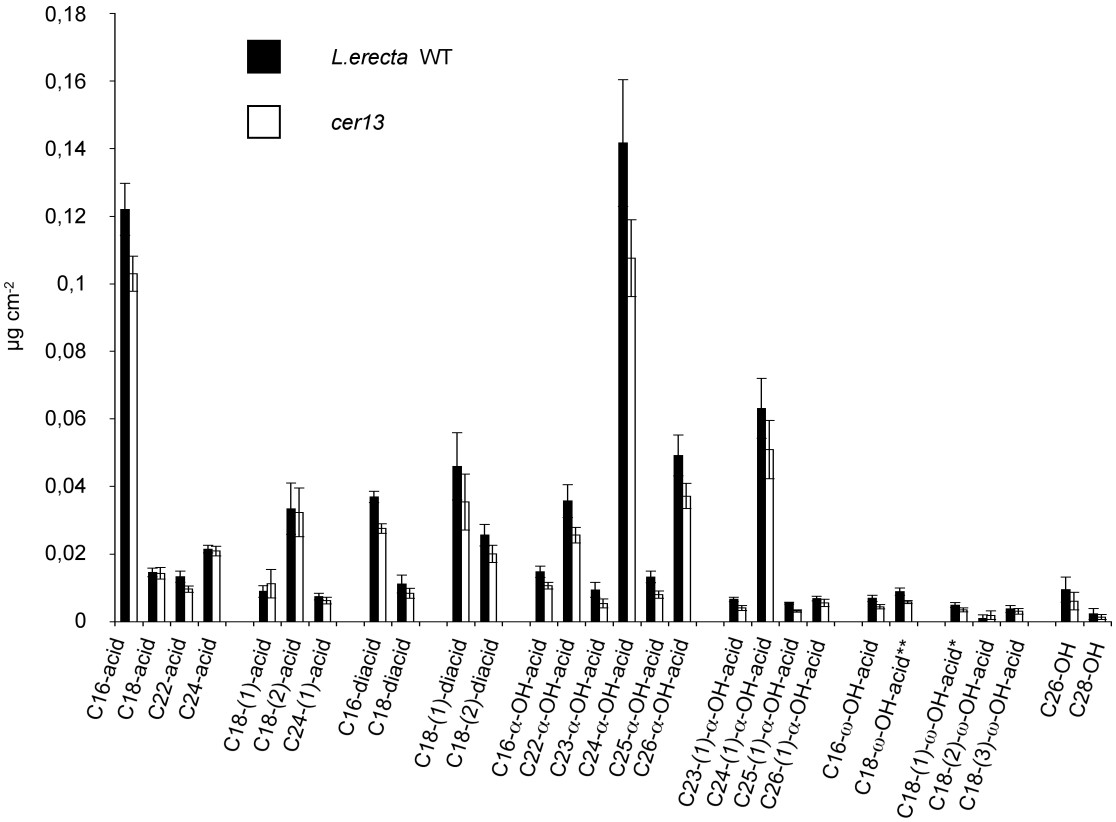

Fig. 26. Comparison of cutin components of *L.erecta* and *cer13*. * indicates C18-(1)-ω-OH-acid/C16-9/10-OH-diacid where second compound lies underneath. ** indicates C18-ω-OH-acid/C16-10/16-di-OH-acid where second compound lies underneath.

The seed coat analysis of *cer13* seeds was carried out in the same way as with *cer10*. The remnants of an improper crushing were present in this analysis as it contained C18-(1)- and C18-(2)-acids with quite a difference in the 5/6 parallels. The analysis did not disclose any differences in the composition of polymeric lipids of the *cer13* mutant when compared to the wild type seed coat lipids (Fig.27). The knock out of the *cer13* gene did not lead to changes in the polymers in the protected seeds which means that it is very likely not involved in lipid biosynthesis in the polymers of the seed.

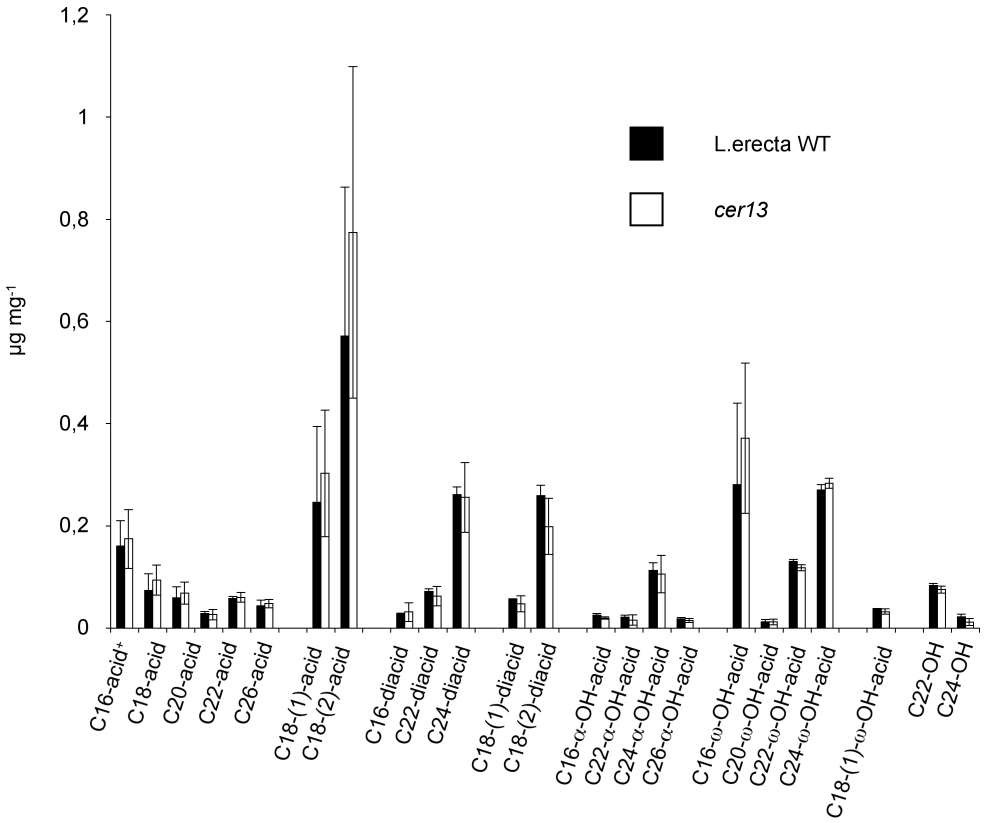

Fig.27. Comparison of seed coat polymeric lipids of *L.erecta* and *cer13*.

3.5. Analyses on *cer3*

The wax composition of *cer3* has been published in Hannoufa et al (1993). It shows the reduction of waxes in the *cer3* mutant in all alkanes, in most alcohols, especially the longer once as well as in the acids with a longer chain length. We conducted wax analysis on *cer3* in comparison to an insertion line which carries an insertion at the end of the putative *cer3* gene (At5g02310). The plants were provided by A. Bachmair (MPIZ Köln, Department for Plant Developmental Biology). The *cer3* plants were homozygous for *cer3* because they exhibited the glossy phenotype. Heterozygous plants were taken as a control and these were chosen from the F1 after crossing Col-0 X *cer3*. The wax bloom of homozygous *cer3* was compared to homozygous GABI-Kat lines (checked for homozygous insertion by A. Bachmair) with the insertion in At5g02310 and this was also compared to the F1 of the cross GABI-Kat line X Col-O wild type. The wax analysis confirmed the previous results for the *cer3* wax bouquet but clearly showed that the homozygous insertion line did not have a reduction in the wax load but it rather resembled the wild type (Fig.28).

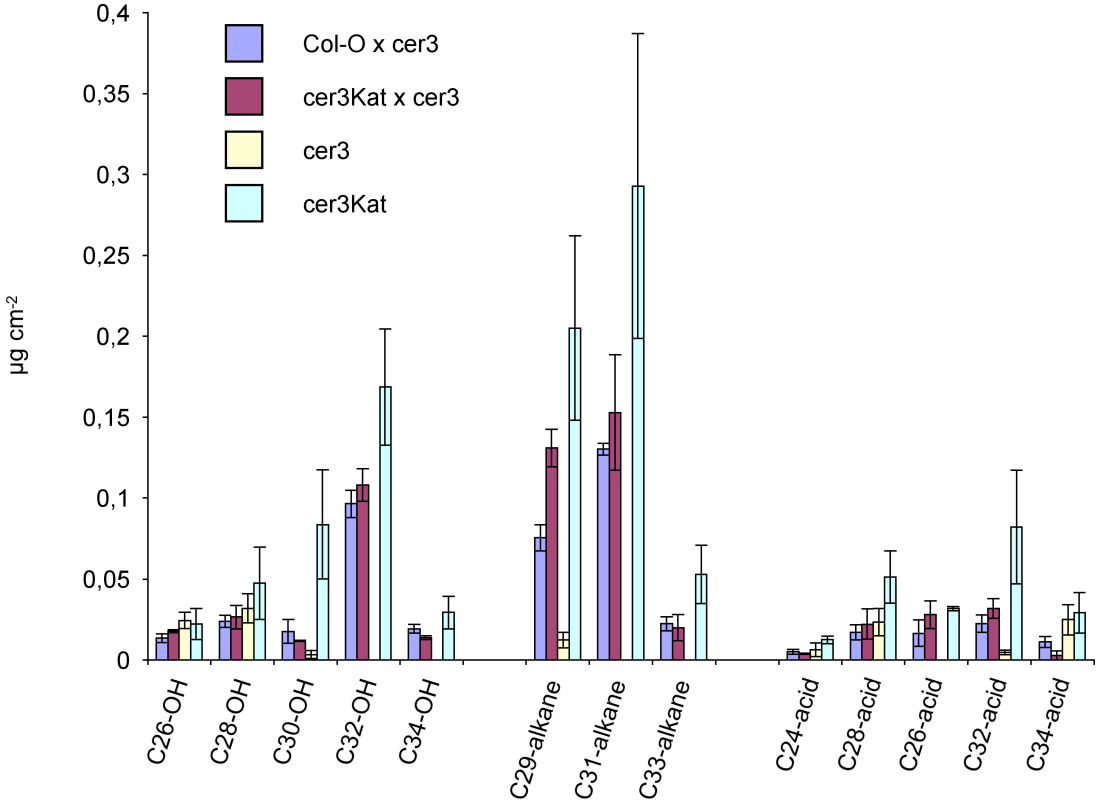

Fig.28. Wax load of Columbia wild type, *cer3* and insertion line in At5g02310 (here called cer3kat).

To genetically confirm the discrepancy of At5g02310 not being *cer3* the F2 of the crosses between GABI-Kat and *cer3* were sown for selection on grodan mats (Hadi et al., 2002) with sulfadiazine as antibiotic. The waxless mutant *cer3* was crossed to the Gabi-Kat line with a known insertion at the end of the putative *cer3* gene (At5g02310). The genetic background of *cer3* was *L. erecta* and of the Gabi Kat line Columbia. After the sulfadiazine selection resistant plants were checked for their waxy phenotype. Approximately 2500 seeds were sown on grodan mats of which around 650 plants grew under the selective conditions. 313 plants were transferred into pots with soil and out of these 62 plants exhibited the waxless phenotype and the remaining 251 were Columbia wild types. This indicated that At5g02310 was not the reported *cer3* gene since all F1 plants would have had the glossy phenotype as the two loci were allelic. From the wax analysis it is clear that no other analysis resembled the *cer3* wax load and did not even differ from the wild type wax load at al. Taken together with the results from the crosses and selection on sulfadiazine clearly demonstrated that *CER3* does not have a mutation in At5g02310.

3.6. Cutin and wax analysis in *O. sativa*

When the sequencing of the *O. sativa* genome was completed in 2004 many orthologous genes involved in wax and cutin biosynthesis in *A. thaliana* were identified in the rice genome. In rice, several databases have been established with insertional mutant lines that are available for everyone (Jeong et al., 2006).

A mutant was identified in T-DNA insertional lines in japonica rice (Jeong et al., 2002) and called *wax deficient anther1* (*wda1*). The gene encodes a protein with a high similarity to fatty acid desaturases such as *CER1* and *WAX2* in Arabidopsis. The mutant *wda1* exhibited morphological changes in pollen as well as in anther walls and additionally it was infertile. The segregating *wda1* plants were tested for the T-DNA insertion and the PCR results were confirmed by GUS staining in the lines carrying the insertion. The T-DNA used for the transformation of rice contained the GUS gene which is genomically inserted and expressed under the promoter behind which it is integrated in the DNA.

To show if *wda1* is involved in wax biosynthesis the analysis of waxes was applied on *wda1* leaves and leaves of the corresponding wild type.

Waxes have been extracted and analysed in many species including rice. Chloroform is used for extraction of all soluble wax components and it is carried out at room temperature for 6 (Welker *et al*., 1998) to 60 (Bianchi *et al*., 1979) seconds. The extraction time is dependent on membrane lipid content and impurities of the extracts. Depending on wax, membrane composition and structure, the membrane components are more or less readily extractable with the wax compounds. In order to compare rice Wt and the *wda1* mutant the wax extraction was carried out for 10 seconds to avoid contamination of the wax fraction with sterols and other components of the plasma membrane. The analysis of the rice waxes was carried out in two extraction steps, at room temperature and at 35°C, respectively. It had been published that rice waxes contain large amounts of aldehydes that require an extraction at higher temperatures. The analysis revealed that the extraction at 35°C was not sufficient to release aldehydes from their clusters and the aldehydes only appeared in the preparation of the cutin monomers for which the remains of the

leaves after the extraction of waxes were used. As aldehydes cannot be bound in the cutin polyester they likely belong to the waxes and I included them in the wax table. The overall wax amount did not reveal differences between the waxes on *wda1* and the wild type (Fig.29).

In previous wax analysis of rice leaves wax contents of 0,23 to 1,41 µg cm^{-2} for different varieties were reported by Welker *et al* (1998). Other values for the wax content of the 3 major peaks ranged from 0,2 to 1,9 µg cm^{-2}. The values for our analysis for the extraction at room temperature was for WT and *wda1* around 5 µg cm^{-2}. If the amounts of waxes from the extraction step at 35°C and the aldehydes from the cutin analysis were added it was around 7,8 µg cm^{-2} for the mutant and the WT.

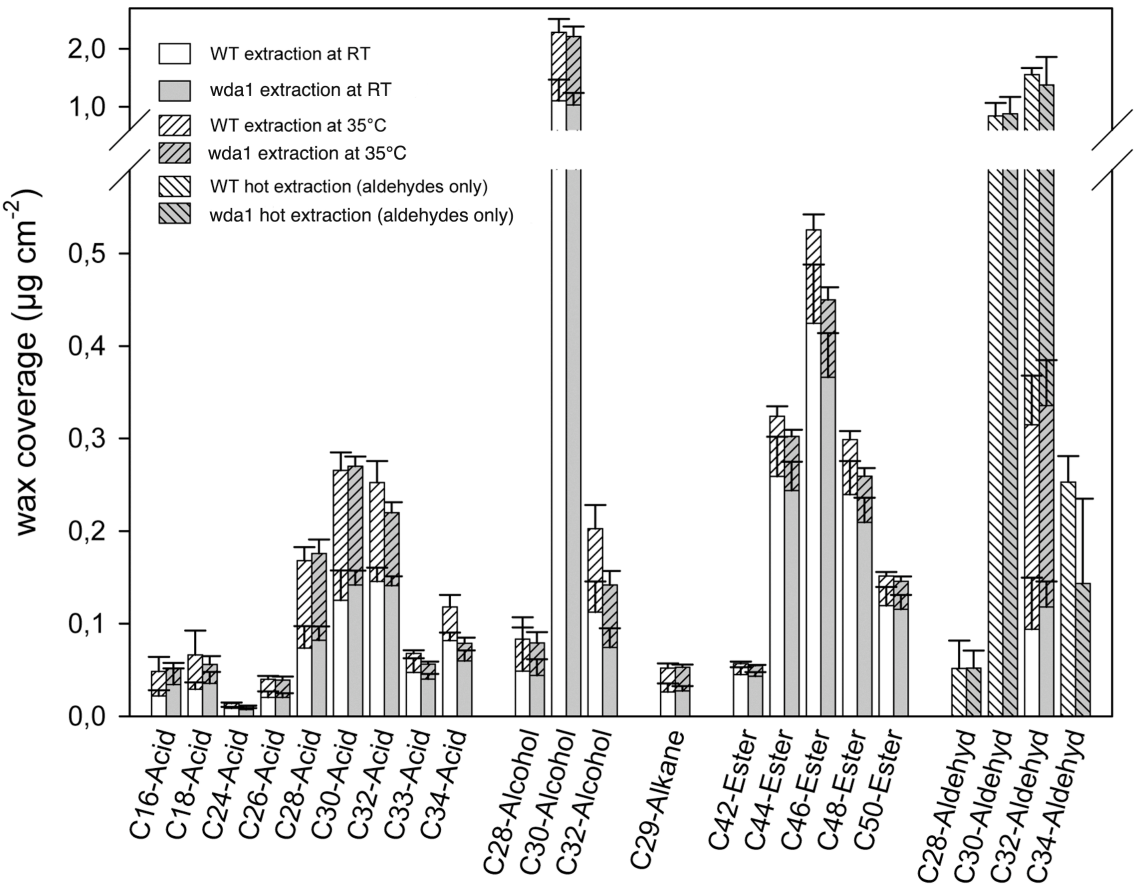

Fig. 29. Rice waxes in the japonica wild type and *wda1* from two extraction steps (at RT and 35°C) and the aldehydes from the extraction for cutin analysis

This result was in accordance with the GUS expression studies conducted on

the *wda1* mutant (Fig.30.) in which the expression of GUS under the *WDA1* promoter was clearly defined to the inflorescences and there at this developmental stage specifically to parts of the anther, the anther filaments, the style, parts of the ovule and the edge of the panicles (not shown).

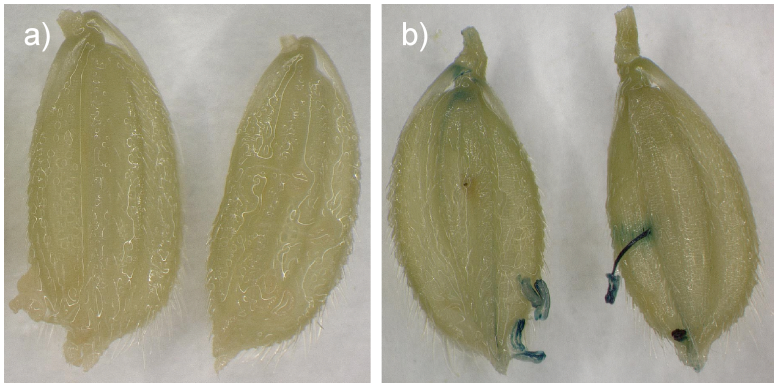

Fig.30. GUS Staining for glucoronidase (GUS) activity in the wild type and *wda1* in the rice flower.
a) No GUS activity was detectable in wild type flowers b) The GUS activity was prominent in anthers besides expression in other parts of the rice flower in *wda1* because the promoterless GUS reporter gene is activated upon T-DNA insertion in *WDA1*.

An analysis on the cutin components did not reveal a change in cuticle composition between wda1 and the wild type (Fig.31). The method applied to *O.sativa* was the same as in *A.thaliana* and the fatty acid composition of the rice cuticle has been estimated for the first time. Although it revealed that *wda1* was not a cutin or wax mutant the composition of cutin in rice is known now. The total amount of wild type *O.sativa* cutin was 3,216 (+/- 0,015) µg cm^{-2} which is five times the amount of *L.erecta* cutin in Arabidopsis. The major components are VLCFA, VLC alcohols, α-hydroxy fatty acids and ω-hydroxy fatty acids. Aldehydes were analysed by this method but they are believed to belong to the wax fraction (Haas et al., 2001; Welker et al., 1998). But the incubation of leaf material with MeOH/HCl at 80°C for 2 hrs could release them from their aldehyde complexes (Haas et al, 2001; Welker et al, 1998). As the cutin composition of *O.sativa* is now known and easily assessable it will be easier to find and analyse cutin mutants in rice and analyse the biosynthesis of cutin components in species different from *A. thaliana*.

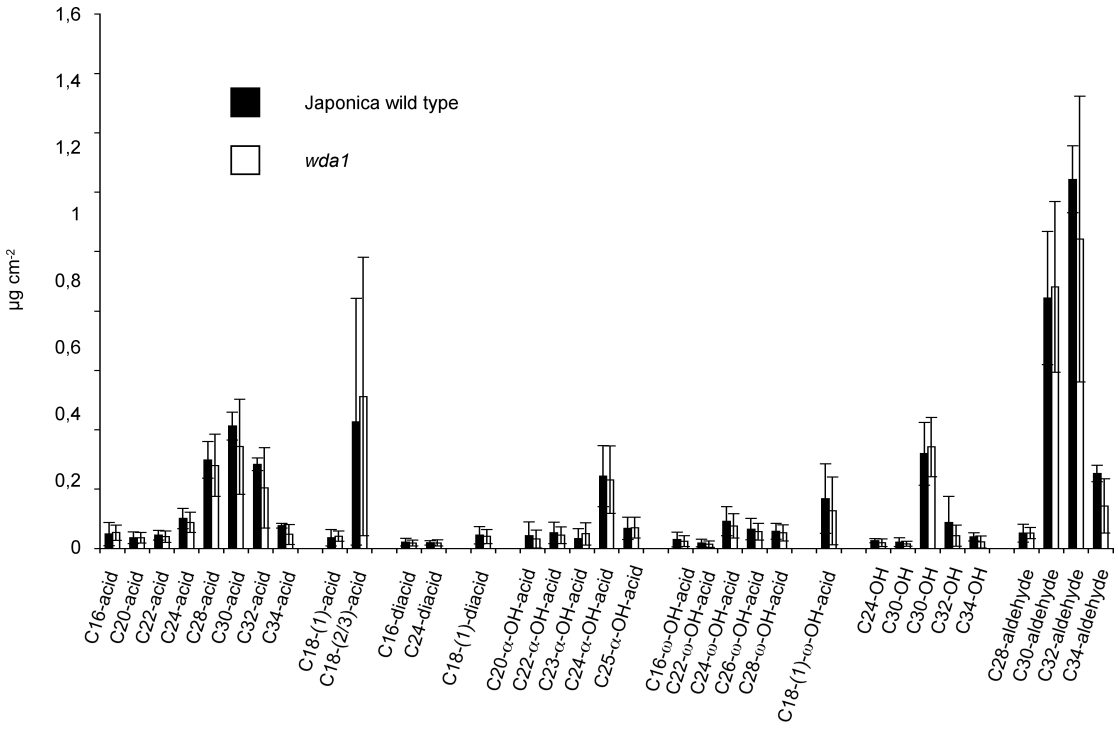

Fig.31. Comparison between cutin in Japonica wild type and *wda*.

3.7. Analysing wt exposed to drought stress

Bonaventure et al (2004) reported the analysis of the cutin composition of totally extracted leaves at 4, 5 and 6 weeks after germination and they could not detect any substantial differences in polyester composition or monomer load at the different stages. We analysed *L.erecta* at different developmental stages: 4 weeks, 4 weeks plus 5 days, and 4 weeks plus 12 days and a difference in almost all components could be seen. There is an increase in all compound classes. The increase is equal to all chain lengths and in all compound classes. One week after the first harvest the amount of cutin components was approximately the same as before (overall change 6,2 % increase). One week later, the amount was already 50,7 % more than at the first time point. Greater increases in the cutin composition and amount were found on plants exposed to drought conditions. *L.erecta* leaves were harvested when they were 4 weeks old and than the plants were not watered at all and leaves were harvested after five days and after another 7 days. In comparison to the 4 weeks old plants after five days the amount of cutin was approximately the same as before (96,2 %) but after 12 days without water the cutin composition increased by 74,3 %. The watered trays and the trays containing the drought exposed plants sat next to one another in the green house, so they were grown under identical conditions apart

from the watering. Exemplary for all cutin components the amounts of diacids are summarised in a table showing the results of cutin analyses from 4 weeks old *L.erecta*, 5 and 12 day later, and the corresponding *L.erecta* that were not watered (Fig.32). The major increases in amount per area unit during the growing of the leaves were found in the α-hydroxy acids (in growing *L.erecta* 60,6 % increase after 12 days compared with 4 weeks old plants and 95,4 % in 12 days not watered plants compared to the 4 weeks *L.erecta*). Likely that this is the reason why Bonaventure et al (2004) did not see a huge increase in developing leaves as they did not recognise α-hydroxy fatty acids in the Arabidopsis cutin although even without the α-hydroxy fatty acids there is still an increase in cutin components per area unit in normal watered plants after 12 days by 31,8 %.

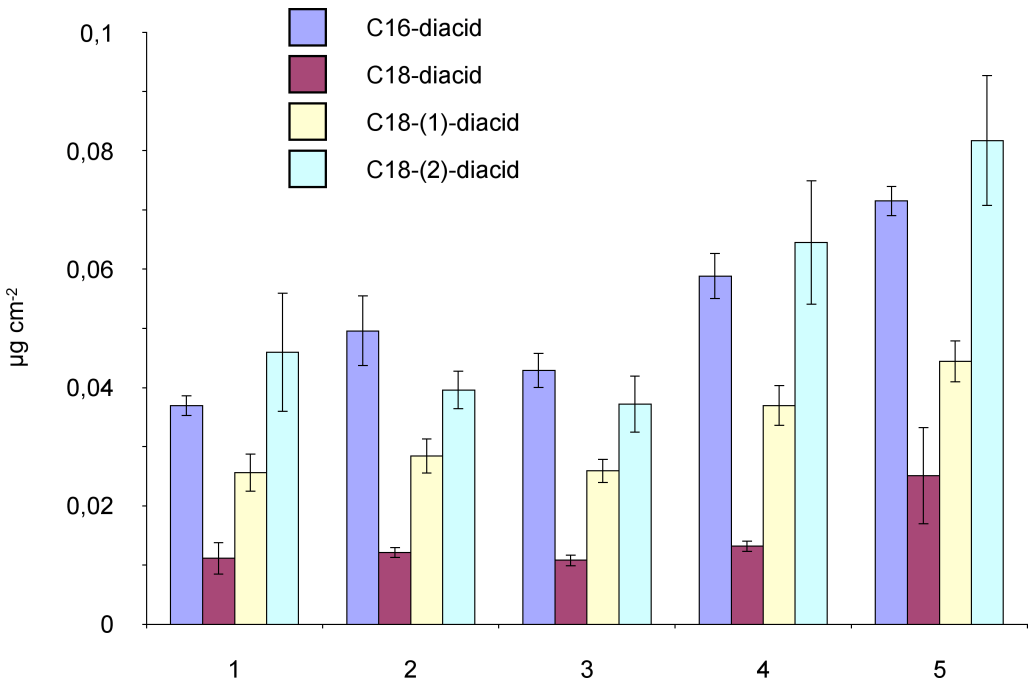

Fig.32. *L.erecta* plants (1) exposed to drought conditions after 5 days (3) and 12 days (5) compared to normally watered L.erecta plants after 5 days (2) and after 12 days (4). For overview purpose only diacids are presented out of the total cutin analysis

4. Discussion

4.1. Cutin

Cutin is the major component of the cuticle and is essential for the plants' protection against the environment. The composition of this protective coating has been known for a variety of plants for a long time (Kolatakutty, 1981 & 2001, Holloway, 1982) but the biosynthesis and transport of the components still needs to be completely discovered.

Arabidopsis thaliana cuticular mutants represent a perfect system to enlighten the synthesis of cutin components and their transport. Besides these insights direct or indirect effects of the cutin composition and of the lipids in more general terms will shed light onto signalling processes, cell-cell communication and plant development.

4.2. The method for cutin analysis

The traditional method for the analysis of cutin components involved the peeling of the cuticle from the organ and the digestion of it in an enzymatic solution (Schönherr and Riederer, 1986). This method was applied to the cuticles of Arabiodopsis but the application was difficult (Xiao et al., 2004, Bonatventure et al., 2004, Franke et al., 2005). So alternative methods were published including cutin analysis without prior peeling of cuticles (Bonatventure et al., 2004, Franke et al., 2005).

Xiao et al (2004) isolated enzymaticly Arabidopsis stem cuticles and determined cutin characteristic depolymerization products, including C16-hydroxy-acid, C16-9/10-16-dihydroxy-acid, C16- and C18-α/ω-diacids. Bonaventure et al (2004) has determined the same components as majors in cutin as us (Franke et al., 2005) including unsaturated C18-α/ω-diacids. For these analyses solvent extracted Arabidopsis leaves were used. These results were supported by the analysis of epidermal peels that were analysed by Bonaveture et al (2004) and by Franke et al (2005).

The analysis of pure cuticles by Xiao et al (2004) the total extraction was carried out by refluxing in chloroform/methanol (1:1) for 24 hours, subsequent depolymerisation of the cuticle with 14% BF_3 in anhydrous methanol to produce methyl esters. These could then be extracted with diethyl ether as already described in Riederer and Schönherr (1986). Derivatisation was carried out using N, O-bis (trimethylsilyl) trifluoroacetamide and the samples were than incubated for 30 min at 70°C. Samples were analysed on GC-FID. The analysis revealed for the Columbia wild type C16-diacid (49,3 %) and C16-10,16-diHydroxy-acid (16,4 %) as major components. The total amount of cutin components on the Columbia stem surface was 0,491 µg cm^{-2} (+/- 0,0082).

In the method used by Bonaventure *et al* (2004) whole Arabidopsis tissues or epidermal peels were extracted prior to the depolymerisation. The ester bonds were cleaved by hydrgenolysis with lithium aluminium hydride ($LiAlH_4$) and the carboxy groups were reduced to their primary alcohols. Other functional groups, such as aldehydes, ketones and oxiranes were reduced to their primary or secondary alcohols. Subsequent analysis revealed all hydrogenolysate products to be C16 and C18 polyhydroxy alkanes and alkenes. The most abundant product in leaves was C18-(2)-1,18 diol. To discriminate between the origin of the different hydroxyl groups the authors employed an isotopic labelling by deuteriolysis with lithium aluminium deuteride ($LiAlD_4$) to the extracted tissues. The authors applied another method on extracted leaves to verify the results of the hydrogenolysis and the deuteriolysis. They used sodium methoxide ($NaOCH_3$) in methanol for transmethylation of the cutin esters with subsequent silylation of the products. The major product in the leaf cuticle was the methylated C18-(2)-diacid.

We published a method (Franke et al., 2005) similar to the one reported by Bonaventure et al (2004) in which total leaves were first extracted in chloroform and by that exempted from all soluble lipids and other soluble metabolites from the leaves. The remains were than depolymerised. In Franke et al (2005) it was shown that cutin components found by that method were the same as cutin components detected in isolated cuticles from a very labour intensive experiment which was once conducted to see if there was a difference between the two methods. The

comparison of different depolymerisation methods (MeOH/HCl and BF_3/MeOH) with different extraction solvents (hexane and chloroform) showed that both methods reveal the same cutin components but with BF_3/MeOH and the extraction with chloroform more carbohydrates were extracted and the method was more time consuming than the depolymerisation with MeOH/HCl and extraction with hexane (Figure 2). So the method of choice for the application to a variety of mutants with a putative defect in cutin biosynthesis was transesterificaition with MeOH/HCl and extraction of lipophilic compounds with hexane.

Using this approach several mutants were analysed and for some a function of the gene could be estimated due to their cutin composition and additional experiments.

Table 3. Methods to analyse cutin components.

Biochemical method	Reference:	Comments
Biochemical analysis of cuticle	Xiao et al., 2004	1. Difficult to apply to Arabidopsis 2. Quite time consuming, 3.Only alcoholic products 4. No quantitative result 5. Differences in mutants are detectable
Biochemical analysis of peeled cuticles and all polymers	Bonatventure et al., 2004	1. Large scale not possible 2. α-hydroxy fatty acids were not detected 3. Quantitative results are measured 4. All polymers of the tissue are analysed or pure cuticles
Biochemical analysis of all polymers	Franke et al., 2005	1. Quantitative results 2. Applicable to mutants with fragile cuticles 3. Similar composition as pure cuticles

Besides this analytical approach other methods for the analysis of the effects on a mutant with a defect in the cuticle have been applied to various mutants as described in the introduction. One thing holds true for all methods: if a mutant appears not to be a cuticular mutant for one method it is not necessarily a cuticular mutant. For example according to Xiao et al (2004) the cuticular mutant *att1* seems to have a reduced amount of cutin components on stems (down to 30 % of wild type) and induces type III genes in *Pseudomonas syringae* but the proven cuticle mutant *wax2* does not enhance the expression these genes in the pathogen. Hence, this pathogen test does not hold true for all cuticle mutants as seen in *wax2* (Xiao et al.,

2004). Another example for the non-universality of the cuticle deficit feature is the fusion phenotype itself. Some known cuticular mutants do not exhibit organ fusions (e.g. *pel4*, *pel5*, *pel6*, Tanaka et al., 2004) or the organ fusion is just restricted to specific organs (e.g. to petals and stamens in *ace/hth*, sepals in *cer10*) although it has been shown by the biochemical analysis of these mutants that they have a non-wild type cutin composition (Fig.5 and Fig.18). Others have the same composition of cutin components as the wild type and almost the same amounts of them but exhibit organ fusions, e.g. *pel3* (Fig.9), *wax2* (Franke et al., personal communication). The postgenital organ fusions have also been known for plants expressing a fungal cutinase (Sieber et al., 2000). These lines extracellularly overexpress a cutinase that digests the cutin polyester which leads to non-separable organs when they touch. The composition of the cuticle of this mutant would be very interesting.

TEM seems to be a good method to look at organ fusion mutants. Putative disruptions in the cuticle in some mutants could be confirmed by their TEM images (eg. *ace*/*hth*, Kurdyukov et al., 2006; *bdg*, Kurdyukov et al., 2006; cutinase expressing plants, Sieber et al., 2002). Normally the cuticular layer is an electron-dense, opaque layer on the outer side of the epidermal cell wall. The fusions in *ace*/*hth* occur in the inflorescences and there the TEM pictures revealed that the cuticle is occasionally discontinous and sometimes multilayered. The biochemical analysis of cutin of leaves seemed to again confirm this cuticular deficit in *ace/hth*. In the *bdg* mutant the TEM pictures show a thicker, less dense but very often the cuticular layer is multilayered on the outer side of the epidermal cell wall on leaves. The biochemical analysis corroborated the finding of the thicker cuticle: the amount of cutin components is elevated 2 to 3 times. However, the relation between the cuticle structure and these organ fusion mutants is not clear. Xiao et al (2004) published TEM pictures of *att1* in which the leaf cuticle looked two times thicker than that in the wild type leaves and also less opaque. But the biochemical analysis that the authors conducted on the stem of the *att1* mutant and on the corresponding wild type stem exhibited a reduction in the mutant of 70 % that is contradicting to the pictures from the cross sections. An explanation could be that there is a difference between the cutin amount and composition on leaves and stems or any other organ of the plant. This has not sufficiently been analysed as Xiao et al (2004) only analysed stems and

we only analysed leaves up until now. Additionally Bonaventure et al (2004) and we seem to have a more precise method for the analysis of cutin components as we both find more cutin components and in greater amounts than Xiao et al (2004). The other explanation for increased thickness of the cuticle in *att1* could be due to the lack of α-ω-diacids in their analysis. As *ATT1/Cyp86A2* encodes a fatty acid ω-hydroxylase it is likely to be involved in biosynthesis of α-ω-diacids (Kurdyukov et al., 2006; Franke et al., 2005). However, because the major components of Arabidopsis cutin, α-ω-diacids (C18) have not been detected by Xiao et al (2004) in their samples, these data should be taken cautiously.

4.3. Alternative analyses: seed coat lipids, wax and suberin

VLCFAs are produced for various pathways and take over many functions. Apart from the cutin analysis, all biochemical clues about the composition of other polyesters or lipids in a mutant will provide information about functions of genes and their involvement in the regulation of the VLCFA biosynthesis. In the lab we started analysing seed coat polyesters as developing seeds are potentially not strongly affected by secondary effects from the environment because they are enclosed in siliques. Additionally we find occasional visible phenotypes on seeds (Fig.12, 15 and 24).

In the seed coat lipid analysis we aimed to identify cutin like lipids in the seed coat but not the storage and membrane lipids. Membrane lipids usually comprise glycerolipids (phosphoglycerides) that contain almost exclusively C16 and C18 unsaturated fatty acids. Storage lipids generally consist of triacylglycerols (TAGs) with varying fatty acid composition (Millar et al., 2000). The polyesters obtained for the seed coat lipid analysis were believed to occur in the seed coat only, as the interior of the seed mainly contains storage lipids and embryo membrane lipids. In the analysis after the crushing of the seeds and thorough extraction of soluble lipids, mainly cutin monomer like lipids rather than membrane or storage lipids were detected and we presume that these are from the seed coat. Unfortunately the evaluation of the surface of seeds used for each sample was not possible so that the results of the analysis could only refer to µg mg^{-1}. The seed coat lipid analysis

conducted on *cer13* revealed no differences between mutant and wild type (Fig.24) whereas the seed coat analysis of *cer10* showed a skewing of seed coat monomers to shorter chain lengths (Fig.15). Because CER10 appears to be involved in the FAE complex, this primary effect seen on lipids in the seed coat on elongation of VLCFAs is in good agreement with the previous hypothesis for the CER10 function (Zheng et al., 2005). Therefore, the seed coat analysis may be very informative and should be applied on other putative cuticular mutants to obtain more information on rather primary effects on the biosynthesis of polymeric lipids.

The analysis of suberin has been established in Arabidopsis recently (Franke et al., 2005) and provides a useful method to analyse the composition of the suberin polyester in roots. The Arabidopsis suberin has a similar composition as cutin and the biosynthetic pathways of cutin and suberin might be related. Therefore the analysis of suberin in cuticular mutants might give new insights in the biosyntheses of both polyesters but have not been carried out yet.

The wax analyses carried out on wax and cutin mutants (Rashotte et al., 2004; Rashotte et al., 2001; Hannoufa et al., 1993) did not lead to gene functions in all mutants analysed. Apparently the wax composition of mutants revealed unspecific changes when compared to the wild type. This can be caused by secondary effects or compensatory events in the biosynthetic pathway or in other cases the missing enzymes are redundant and the missing function of the gene cannot be detected by the analysis of the wax.

4.4. Mutants with a defect in the cutin biosynthesis

We applied the newly developed method for the analysis of cutin components of very fragile cuticles to a number of cuticular mutants in order to elucidate the functions of the respective genes some of which were already cloned others not. The analyses of two mutants lead to an idea of the function of the two genes.

The *ace* gene is allelic to *hth* and APB24 and it could be shown that it is epidermis specifically expressed (Kurdyukov et al., 2006). Organ fusions occur in ace in flowers only and plants are infertile. The cutin analysis showed a reduction in

diacids (81,4 % of the wild type) and an increase in ω-hydroxy fatty acids (139,8 % of the wild type) (Fig.5). Believing that the *ace* mutant is impaired in the ω-oxidation pathway there must be similar enzymes taking over parts of the ACE function as the pathway is not completely blocked. The results from the cutin analysis corroborates the analysis of all extractable lipids (Kurdyukov et al., 2006) in which ω-oxo fatty acids are reduced and the ω-hydroxy fatty acids accumulated in the same way as seen in the cutin monomers for *ace*. If these results are no secondary effects it might be true for ACE to be involved in the ω-hydroxylation pathway (Fig. 31). Considering the next step in the ω-hydroxylation pathway an aldehyde dehydrogenase might be responsible for the subsequent oxidation of ω-oxo fatty acids to α/ω-diacids. The analysis of waxes in *ace* showed no differences to the wild type (Fig.6). Concerning the hypotheses about the remedy of the genetical deffect detected by Lolle et al (2005) the function that we propose does not explain it.

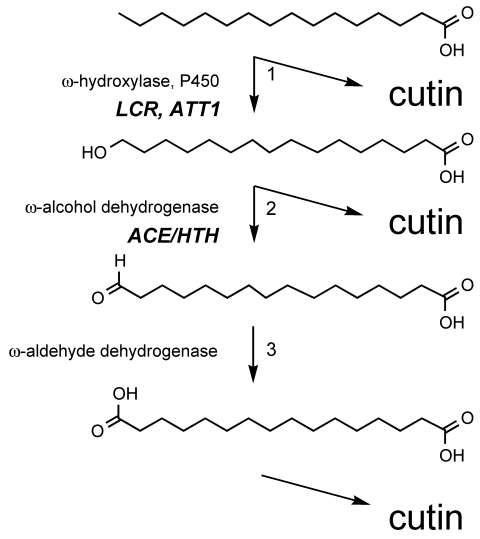

Fig.31. Possible involvement of ACE in the ω-oxidation pathway in the biosynthesis of cuticular monomers (Kurdyukov et al., 2006).

A strong organ fusion phenotype is seen in the *bdg* mutant. *BDG* is expressed similar to *ACE*, epidermis specifically, and this has been shown by promoter fusions to GUS and GFP, and in situ hybridisation (Kurdyukov et al, 2006). The sequence of the *BDG* gene shows homologies to α/β-hydrolase containing proteins which are responsible for the catalysis of a variety of reactions. These proteins include lipases, esterases, epoxide hydrolases and acyltransferases but BDG belongs to a specific family of which the function is yet unknown and might be novel. Cutin analysis was

conducted on *bdg* and the cutin of this mutant consisted of two to three times more cutin components than the wild type. The same phenomenon was true for the wax content on *bdg* (Kurdyukov et al., 2006). Believing that BDG is not directly involved in the biosynthesis of cutin monomers the results propose a function in the esterification of cutin monomers outside of the cell wall. Furthermore the results suggest a very strong compensatory effect for wax and cutin components as all amounts are increased two to three times (Fig.7). The mutant seems to upregulate all pathways to ensure the integrity of the cuticular layer (wax and cutin) as a secondary effect of the improperly connected pieces of cutin or cell wall component in the *bdg* mutant.

Other mutants exhibited unspecific changes in their cutin components. For example the transgenic FDH:FAE plants, expressing *FAE1* under the epidermis specific promoter of *FDH*, showed such unspecific elevations which cannot be easily explained (Fig.8). VLCFAs, ω-hydroxy fatty acids and α-hydroxy fatty acids were integrated into the FDH:FAE cuticle in higher amounts and diacids and alcohols were reduced (Fig. 9). One would have expected according to the normal function of *FAE1* in seeds where it is responsible for the elongation of VLCFA from C20 to C22 (James & Dooner, 1991; Kunst et al., 1992; James et al., 1995; Millar & Kunst, 1997) that the chain lengths of lipids in FDH:FAE would have been longer. But there was no trend in this direction detectable. So the function of *FAE1* in the epidermis was a different one and might have been just an involvement in signalling processes. The further analysis of the FDH:FAE mutant might give insights in the signalling processes that are involved in epidermal development and cell cycle as the FDH:FAE mutant completely lacks trichomes.

The *pel3* mutant exhibited unspecific changes in cutin monomers that could not be traced back to the knock out of the gene that is homologues to transferases (Fig.10). The cutin composition is very likely a result of compensatory effects that changes activities of enzymes in the cuticle biosynthesis and mask the primary effect of the out-knocked or down-regulated PEL3. Interestingly, with the cutin analysis and the knowledge of the cutin composition of *pel3*, the faster permeation of toluidine blue (Tanaka et al., 2004) cannot be explained as all cutin components are either the same as in the wild type or have increased amounts. The additional results showed

that *pel3* has no changes in the wax composition (Fig.11) so there is no compensation in the anabolism of waxes and no compensation of a loss of PEL3, at least not to the extent where the accumulation of specific components was visible. The rather strong seed phenotype which reminds of the *ap2* seed phenotype reveals an involvement of PEL3 in the development of the seed coat either in the formation of the lipophilic polyester forming the secondary cell wall on the seed coat or in the signalling process that leads to the proper development and formation of the epidermis and the seed coat layers (Haughn and Chaudhury, 2005).

4.5. Mutants with a defect in wax and cutin biosynthesis

The wax and fusion mutant *cer10* has some changes in morphology when compared to the wild type (Fig.13). The whole *cer10/ecr* plant is smaller than the wild type and has less shoots. The *cer10* flowers show fusions between sepals, and inflorescences and can grow in a fiddlehead like shape due to the fusion of the flower. The overall glossiness includes stems, leaves, inflorescences and siliques. Siliques show occasional deformations, and flowers have occasionally additional organs (Fig.14). Recently knock-out mutants of the *ECR* gene in Arabidopsis were identified as *cer10* (Zheng et al., 2005), and the authors also described the morphological differences to the wild type similar to the ones we observed. *ECR* has no very close homologue (Zheng et al., 2005) but 5 other genes annotated as steroid 5-alpha-reductase, like ECR, exist in the Arabidopsis genome (Costaglioli et al., 2005). It was thought that ECR is ubiquitously part of the different elongation complexes. Despite that the *cer10/ecr* mutants still produce some amounts of VLCFAs (75,5% of the wild type VLCFAs). In *N. benthamiana* the T-DNA knock-out mutant of *ECR* produces plants that are embryo lethal and not viable (Park et al., 2005). The disruption of *ECR* in *NbECR* by virus-induced gene silencing (VIGS) lead to a reduction in the VLCFA content in the plants and an increase in fatty acids with less than 18 carbon atoms (Park et al., 2005). At the cellular level the structure of the plasma membrane and of the chloroplast thylakoid membrane were highly abnormal which the authors interpret in the function of the in the organisation of the membranes (Park et al., 2005). For the milder *ecr/cer10* mutant in Arabidopsis aberrant endocytic membrane trafficking and defective cell expansion has been reported, so the knock-out of *ECR* still has a

strong effect on the membranes but it is not lethal as in *N. benthamiana*. So likely one of the candidates mentioned in Costaglioli et al (2005) can compensate parts of the *ECR* function in Arabidopsis.

The cutin analysis revealed a strong secondary effect in the cutin composition in *cer10/ecr*. Major changes in the composition were detected in the reduction of α-hydroxylated fatty acids (69 % of the wild type) and long chain alcohols (~ 64 % of the wild type) (Fig. 17 and 18). When the chain lengths are compared there is not a skewing to shorter lipids as would have been expected for a mutant with a defect in the elongation complex. To look at a more direct effect of the knock-out of *ECR/CER10* the seed coat lipids were analysed because this polyester is produced when the seed is protected from environmental effects as they are enclosed in siliques. The seed coat lipid analysis showed a clear accumulation of lipids with 16 carbon atoms, C18 lipids are 92 % of the wild type and with increasing chain length the amounts in the *cer10* mutants become smaller (C20: 78 %, C22: 56 %, C24: 42 %) (Fig.19 and 20). But there is not a total lack of VLCFAs that show again the redundancy of *ECR*.

The compensation reaction in the composition of the cuticle shows a reduction of α-hydroxy fatty acids.

In the literature only a few enzymes have been reported to be involved in the α-hydroxylation process. For Arabidopsis there was only one publication about α-hydroxylation (Mitchell et al., 1997). A close homologue in Arabidopsis to an α-hydroxylase in *S. cervisea* has been reported to be able to complement the gene in *S. cervisea*. In the report Mitchell et al (1997) were investigating a cytochrome b_5-like gene located on the S. cervisea chromosome XIII at locus YMR272C (FAH1). The b_5 core for which the gene encodes exhibits a 54% similarity to the well characterised b_5 core domain of OLE1 a *S. cerevisiae* Δ 9 fatty acid desaturase. The yeast strain lacking the *FAH*1 gene does not exhibit an obvious phenotype. Upon analysis of the most abundant long chain fatty acids (C14 to C18) no differences were observed in comparison to the wt. But when sphingolipid-derived very long chain fatty acids were analysed an approximately 40-fold reduction of α-OH 26:0 fatty acids and a

complementary increase in 26:0 fatty acids were revealed. These fatty acids only occur in minor amounts but are physiologically important as they are incorporated into the sphingolipids. In sphingolipids, the hydrophobic ceramide portion is composed of the long chain base phytosphingosine, which is amide linked to the VLCFA. The majority of these fatty acids in sphingolipids are α-hydroxylated. The major functions of sphingolipids in plants are firstly to serve as membrane structural components, secondly they play a role in signalling and cell regulation, thirdly they take part in cell cell interaction. The *A. thaliana* FAH1 exhibits 42,6% identity and 62,1% similarity to the *S. cerevisiae FAH1*.

A number of motifs found in lipogenic enzymes are conserved besides the cytochrome b_5. The general motif $HX_{(2-3)}(XH)H$ that is characteristic for membrane-bound desaturases can act to coordinate a μ-oxo diiron cluster (Fe-OFe) that functions as a part of a reaction center. Overall 5 of these histidine-rich motifs can be found in the *A. thaliana* FAH1. With a hydrophobic analysis Mitchell et al (1997) predicted YMRC171C (FAH1) and its homologues have two transmembrane regions both as hydrophobic domains that are sufficient to pass the membrane bilayer. These properties suggest in comparison to OLE1 that the active site is assembled from the histidine-containing motifs on or near the membrane surface. All evidences lead to the assumption that *FAH1* in S. cerevisea and its homologue in *A. thaliana* functions as an α-hydroxylase. Microarray analyses of stem epidermis showed that the expression of *FAH1* and *FAH1-like* are not elevated in the Arabidopsis stem epidermis (Suh et al., 2005) and there are no reports of *fah1* or *fah1-like* mutants that exhibit an organ fusion phenotype.

The expression analysis of *FAH1* and *FAH1-hom* showed that the expression of these two genes is not upregulated to an extent that would be visible by RT-PCR (Fig. 21). Other genes might be involved in the production of α-hydroxy fatty acids for the cutin biosynthesis and upregulated in the case of *cer10/ecr* or in *cer10/ecr* α-hydroxy fatty acids are channeled to the pivotal cutin biosynthesis from other pathways like sphingolipid biosynthesis to prevent the formation of an imperfect cuticle. Glc-Cers were analysed by Zheng et al (2005) but differences were minimal in the *cer10* mutant. The analysis of all hydroxylated fatty acids in *ecr/cer10* revealed

an increase for the ones with16 carbon atoms by 20% at the expense of hydroxylated VLCFAs. Other sphingolipids are supposed to be even more abundant than Glc-Cers but the actual composition of Arabidopsis sphingolipids has not been established yet. A proper analysis of α-hydroxy fatty acids in sphingolipids would be needed to solve the question if the reduced amounts of α-hydroxy lipids in the cuticle are due to a change in the amount of sphingolipid α-hydroxy fatty acids. If this was so, the morphological changes in *cer10/ecr* could be caused by the change in sphingolipids as they function in the establishment of proper membranes and in maintenance of cell polarity and cell cycle progression through signalling.

Plant sphingolipids consist of a ceramide backbone with an additional polar head group which can be various glycosyl residues or else phosphate-containing headgroups (Sperling et al., 2005). The ceramides are composed of long chain bases (2-amino-1,3-dihydroxyalkane) carrying a N-acylated fatty acid of 14-26 carbon atoms. The basic ceramide structure and the polar headgroup are highly variable in their chain length, methyl branching, insertion of additional hydroxyl groups and degree of unsaturation (Sperling et al., 2004). The α-hydroxy fatty acids detected in Arabidopsis cutin are the same as the acyl chain of ceramide derived membrane lipids (Lynch and Dunn, 2004). Franke et al (2005) speculated if the α-hydroxy fatty acids function as hydrophobic end caps of the cuticle and therefore are more readily extracted and not seen in great amounts in the enzymatically extracted cuticles. Another hypothesis of Franke et al (2005) was that the amphiphatic α-hydroxy fatty acids could act as cutin primers being anchored in the plasma membranes with their lipophilic tail having the polar head group as polymerisation site. Also in this scenario the enzymatic production of pure cuticles would prevent all α-hydroxy fatty acids from remaining in the cuticle. As another hypothesis it could be that sphingolipid-rich lipid rafts are putative connections between the plasma membrane of the epidermis and the cuticle through the cell wall.

The wax phenotype of *cer13* is rather weak and only detectable by a slight glossiness on the upper stems of the mutants. Fusions in *cer13* have been reported but were not detected under our greenhouse or in tissue culture conditions (Yephremov and Schreiber, 2005). Morphological changes in *cer13* include a bushy

appearance with more branches than in the wild type (Fig.23). This resembles the bushy phenotype of two organ fusion mutants, *lcr* and *bdg* (Wellesen et al., 2001; Kurdyukov et al., 2006). Previous wax analysis of *cer13* did not provide evidence for the function of the mutated gene (Rashotte et al., 2001). The cutin analysis showed no real changes compared to the wild type in the content of the long chain fatty acids, but all other cutin components subsisted of smaller amounts (Fig.26). With the additional data of the analysis of the lipids of the seed coat, no clue was provided that could elucidate the function of CER13. The *cer13* seeds showed a difference in shape and patterning of the surface when compared to the wild type (Fig.24). Seemingly no connection between the composition of the seed coat lipids (Fig.27) and the appearance of the seed coat can be drawn. The mapping process by the map based cloning approach of *CER13* lead to a position in the Arabidopsis genome where more SNPs are available and further mapping and a candidate search will be the tools to get nearer to the gene via the recombinant lines (Fig.25). The candidate search might include the sequencing of candidate genes and analysis of respective mutants available from seed stock centres. The selection of candidate genes may be facilitated based on recently published data on genes expressed epidermis specifically in the Arabidopsis stem (Suh et al., 2005).

Two publications reported on the characterisation of the *cer3* mutant and molecular identification of the *CER3* gene (Hannoufa et al., 1996, Eisner et al., 1998). The annotated *CER3* gene (At5g02310) has a nuclear localization sequence and is putatively involved in protein degradation. As the clear involvement of CER3 could not be estimated by its annotation and suprisingly, the T-DNA insertion lines did not reveal the glossy phenotype (Yin et al., 2004), wax analysis was conducted on the original *cer3*, its cross to the T-DNA insertion line and heterozygous controls as wild type. The wax analysis confirmed the previous results for the *cer3* wax bouquet but clearly showed that the homozygous insertion line for At5g02310 did not have a reduction in the wax load but it rather resembled the wild type (Fig.28). A discrepancy could also be shown between the chromosomal localisation of At5g02310 and the *cer3* locus by sowing the F_2 crosses between GABI-KAT T-DNA insertion line and *cer3* for selection. After the sulfadiazine selection we checked resistant plants for their waxy phenotype.

Only a small number of examined plants that grew under selective conditions exhibited the *cer3* phenotype (19,8 %, 62 of 313 plants). From the wax analysis it is clear that wax analysis of the insertion line in At5g02310 did not resemble the *cer3* wax load and did not even differ from the wild type wax load at al. Taken together with the results from the crosses and selection on sulfadiazine we can be sure that *cer3* is not At5g02310. A forward genetics approach supported our findings and it could be shown that *cer3* is allelic to a known gene involved in cuticle and wax formation that is located on chromosome 5 but approximately 20 cM away from At5g02310 (L. Kunst, personal communication).

4.6. Biochemical plasticity of plant cuticles

The cuticle of Arabidopsis is rather thin and the thickness differs between organs: on leaves it is 20 – 25 nm and on stems 50 – 80 nm (Nawrath, 2003). Interestingly not only the size of the cuticle varies in comparison to species with a thicker cuticle but also the composition. Known cuticles contain in the cutin polyester hydroxyl, and epoxy-hydroxy fatty acids with chain length of 16 or/ and 18 carbon atoms whereas in Arabidopsis it was found out that it mainly consists of α- and ω-hydroxy fatty acids, α-ω-diacids and minor amounts of classical cutin components such as dihydroxy fatty acids or hydroxylated diacids (Xiao et al., 2004, Bonatventure et al., 2004, Franke et al., 2005). With these components the Arabidopsis cutin rather resembles a suberin like composition (Kolattukudy, 2001; Bernards, 2002) whose function is to seal the tissue in the roots (Nawrath, 2003). The analysis of wax and cutin components of mutants with defects in the cuticle or wax biosynthesis revealed that apparently the pathways are associated and dependent or influenced by deficiencies in the cutin monomers, cutin structure and wax composition. One example is the lack of *bdg*, an α/β-hydrolase fold protein putatively responsible for the transesterification of cutin monomers, the loss of BDG function causes an increase in the production of cutin components of more than two times. Although the cuticle of *bdg* appears to be really thick on TEM pictures the lack of the improper interconnection leads to the compensatory effect of anabolic pathways of the cutin components. An analysis of the expression of genes that are involved in the cutin biosynthesis should reveal a striking increase in the amount of RNA that has already been detected for genes

involved in the accumulation of waxes as reported by Kurdyukov et al. (2006). Or if these genes will not show enhanced expression they will likely reveal an increased translation rate or a higher protein activity. Further studies on genes and enzymes involved in the pathway will elucidate this phenomenon. The lack of ACE, an enzyme being putatively involved in the ω-oxidation of ω-hydroxy fatty acids, does not seemingly lead to a compensation of the reduction of the products in the reaction and an accumulation of the substrate, at least in leaves. The putative substrate accumulates (ω-hydroxy fatty acids) and the products are reduced (α-ω-diacids). Apart from these two mutants the analysis of the cutin of *pel3*, FDH:FAE, *cer10* and *cer13* could not lead to a proper understanding of the function of the missing or introduced gene or the compensatory effects of the cutin anabolism. Further analysis is required to understand the functions of enzymes in the cutin biosynthesis and the regulation of the biosynthesis of cutin monomers. However, all results lead to the conclusion that the composition and not the thickness of the cuticle or not the amount of waxes determine the properties of the extracellular matrix. The up- or downregulation of parts of the cutin monomer biosynthesis leads to the compensation by the accumulation of one particular class of lipids (like α-hydroxy fatty acids in FDH:FAE) in the cuticle. But the specific function of these cutin monomers and how it may compensate for a fragile and improper cuticle and provide the proper protection of the plant, is not yet known. The same lack of knowledge applies for the fusion itself as a reduction or accumulation of specific cutin monomers does not necessarily lead to an organ fusion in the cuticular mutant. Like in *cer10*, where the α-hydroxy fatty acids are reduced and do not lead to a fusion phenotype in the leaves but only to a more rapidly leaching chlorophyll (Lolle et al., 1996) and a staining with toluidine blue (Tanaka et al., 2004) in terms of “cutin”-phenotypes.

In most mutants there is not even a total lack of the function of the mutated enzyme as a redundant protein takes over parts of the function as seen in *cer10/ecr*. In this mutant the *ECR* is knocked out and still there is a production of VLCFA in Arabidopsis. Likely due to the redundancy of the enzyme a similar enzyme cares for a complete or partial substitution in the pathway. But in the case of *cer10/ecr* the redundant enzyme(s) cannot completely compensate for *ECR* as seen in the analysis of seed coat lipids (Fig.20 and 21) and results of other analyses published by Zheng

et al (2005). The initiation of the compensatory steps still needs to be explored. It could be true that this is not only due to the improper cutin or wax of the mutant but a consequence of the stress that the mutant plant is exposed to like water stress caused by the improper cuticle that the plant than tries to defend by these compensatory pathways.

Bonaventure et al (2004) analysed leaf cutin composition of wild-type Arabidopsis plants at 4, 5 and 6 weeks after germination. They could not detect substantial differences in the polyester composition and monomer load. When I conducted the cutin analyses of wild type plants under normal greenhouse conditions and exposed to water stress I found changes in the cutin compilation, already in the wild type that was watered normally (Fig.32). In the wild type the overall increase in cutin per area unit during the twelve days growths was 50,7 %. For wild type plants that were not watered at all after the first time point (5 days) the increase in cutin components was 74,3 %. The increase in quantity was approximately the same in all chain lengths and compound classes.

The method to analyse cutin from previously extracted leaves was also applied to *O. sativa* japonica. A mutant called *wda1* that carried a T-DNA insertion in a gene orthologous to the Arabidopsis *CER1* and *WAX2* was analysed as a putative cutin and wax mutant. The wax analysis as well as the cutin analysis revealed no significant changes in *wda1* (Fig.29 and 31). It was found out that *wda1* is only expressed in flowers (Fig.30) and this is also where it is active and leads to major changes in the polyester composition (Jung et al., submitted). As the cutin composition of *O.sativa* is now known and easily assessable it will be easier to find and analyse cutin mutants in rice and analyse the biosynthesis of cutin components in species different from Arabidopsis.

The pathway for cutin biosynthesis might be differentially regulated in young and old plants. Genes and enzymes involved could be different ones although related in function. Likely the same phenomenon applies for different tissues. It is possible that different enzymes and transport systems are responsible for cutin build up. This can be seen in WDA1 in rice that is only active in anthers (Jung et al., submitted)

whereas the mutants in *WDA1* homologues in Arabidopsis, *CER1* and *WAX2*, exhibit phenotypes on leaves.

Another example for differential activity of a pathway in the lipid metabolism is the clear difference of the composition of waxes on Arabidopsis stems and leaves. On the C24 wild type on the stem, for example, the total amount of waxes is 30 times higher than on leaves (Chen et al., 2003). The major components on stems are the C30-aldehyde, the C28-alcohol, the C29 alkanes and the C29-secondary alcohol and on leaves the majors are the C29-alkane, the C31-alkane and the C33-alkane (Chen et al., 2003). The same could apply for cutin components that could explain the differences in the analysis of the pure cuticles on stems (Xiao et al., 2004) and on isolated cuticles from leaves (Franke et al., 2005). Likely different requirements by the plant are fulfilled by the various compositions of the cuticle and waxes on different parts of the plants. This means, for example, that on the leaves which are differently exposed to environmental conditions than the stem of the plant, the plant is in the need for a specific compound compilation and assembly to ensure the survival. On leaves the conditions lead to higher evaporation of water from the plant and stronger radiation by UV-light compared to the stem where the surface is rather shed from direct sunlight and heat by the shade of the inflorescences or neighbouring weeds. So the fulfilment of the demand for protection would be perfect when the means are not the same for both organs.

With regard to the cutin composition the analysis of cutin of *L.erecta* plants exposed to drought stress showed increases in all cutin components. Basically the amount of components of all chain lengths and all functional groups increased but a major increase was detected in the amount of α-hydroxy fatty acids. This compensatory effect appears to be similar as in the cutin analysis of FDH:FAE where the same compounds are produced at a higher rate but in drought exposed plants other compounds exist in higher amounts as well. Under drought stress the plants tried to keep the water in the plant by providing a thicker diffusion barrier through the cuticle. Apparently the α-hydroxy fatty acids are more efficient for this purpose than other cutin components in the wild type. But in the plants exposed to drought stress the increases were not restricted to one compound class. These results indicate that

all cutin components are produced and crosslinked to the cell wall at a higher rate with the time. During the time when leaves expand the amount of all cutin components increases equally. The thickening of the cuticle due to drought stress has been mentioned for other species for example during the Mediterranean summer (Grammatikopoulos et al., 1998, Paoletti, 2005). But the authors could not exclude that the increase in the thickness of the cuticle is due to the increase in UV-B radiation. Of rather general knowledge is that species growing under water stringent conditions have thicker cuticles than plants in a rather wet environment. This phenomenon is also true for the wax crystals on the surface of the cuticle. O'Toole and Cruz (1983) reported on rice cultivars growing in wet lowlands and in dry highlands in South America which have differing wax loads. Additionally to the quantity of waxes the authors report of different rice lines that also assemble different wax compositions. An example for a differing wax load in Arabidopsis, conferring a higher drought tolerance is the SHN1/WIN1 overexpressing plants that carry a waxload that is six fold increased in comparison to the wild type (Aharoni et al., 2004). The authors demonstrated that the overexpression of the SHN gene enhances the drought tolerance of the plant and its recovery after a period of water deficiency. But the authors could not rule out the possibility that the reduced stomata index or a change in the structure of the root system and/or a change in suberin deposition in the root could lead to the drought resistance (Aharoni et al., 2004). Other means reported in the literature of protecting the plant of dehydration stress is the overexpression of aldehyde dehydrogenases. These enzymes demonstrate a broad functional spectrum including toxic aldehyde detoxification, inhibition of lipid peroxidation, ROS-scavenging effects and antioxidative properties in order to maintain the plant tissue integrity under abiotic stress conditions (Sunkar et al., 2003). But the reason why aldehyde dehydrogenase overexpressing plants are more resistant to drought stress is not clear yet.

A deeper knowledge of the cutin biosynthesis and its regulation plus a clearer view of the function of other factors in the plants response to stresses such as the up-regulation of the expression of aldehyde dehydrogenases will lead to the understanding of how the plant tries to protect itself in the best possible and efficient way. As water deficit is one of the most prevalent causes of crop yield loss because

of the strong link between transpiration and photosynthesis, insights will help to produce crops with a greater resistance to drought. The knowledge about the involvement of the cuticle in drought resistance will lead to a better understanding that will be beneficial for crop protection.

5. Summary

Plants are protected by the extracellular cuticle, which is made up of cutin, cutan and waxes. The cutin composition of a variety of plants has been known and models of the biosynthesis of cutin monomers exist but not many enzymes have been identified. It is generally accepted that a defect in the cuticle leads to an organ fusion phenotype. In the model plant *A. thaliana* many fusion mutants have been identified but the identification of genes involved have not lead to a complete picture of the biosynthetic pathway as the fragile Arabidopsis cutin could not be analysed so far.

In this thesis a method to analyse cutin in Arabidopsis was developed and different procedures for the analysis were tested. The analysis of totally extracted leaves instead of isolated cuticles was found to be applicable to analyse Arabidopsis cutin. The main lipids in the Arabidopsis cutin were found to be ω-OH-fatty acids, α-OH-fatty acids, α-ω-diacids and VLCFAs. The optimal depolymerisation step was carried out using methanolic hydrochloric acid and the extraction of lipophilic cutin monomers was performed using hexane. After the subsequent derivatisation with BSTFA the cutin monomers were analysed with GC-MS and GC-FID. The application of the method to the fusion mutant *ace/hth* revealed together with other background information to the knowledge that it might have a defect in the ω-oxidation reaction of ω-hydroxy-fatty acids to ω-oxo-fatty acids that are putative precursors for α-ω-diacids that occur in the Arabidopsis cutin.

Another organ fusion mutant that was analysed was *bdg*. BDG shows homology to α/β hydrolases and the cutin analysis revealed an increased amount of all cutin components by two to three times. This was reconfirmed by previous results from other experiments and the putative function of BDG was suggested to be the transesterification of the cutin components in the cell wall. The upregulation of the cutin biosynthetic pathway, which might be the cause for the increased amount of cutin, could be a compensatory effect of the plant trying to complement the improperly built cuticle.

Other putative cuticular mutants were analysed and the cutin composition of *pel3*, *cer13* and FDH:FAE was changed in an unspecific manner. Additional experiments will be necessary to verify the function of the genes as the results of the

cutin analyses were rather due to secondary or compensatory effects than being caused directly by the knock-out (*pel3* and *cer13*) or differential expression (FDH::FAE) of genes.

The *cer10/ecr* mutant was characterised because CER10/ECR is a candidate for the last reduction step in the FAE complex on the cytosolic site of the ER. Defects of *cer10/ecr* were a change in the overall size of the plant, fusions in flowers, a reduced amount of wax on all aerial organs, difference in the sepal surface in comparison to the wild type and occasional organ duplication in flowers. It could be shown, that *CER10* is allelic to *ECR*. So all defects should be caused by a mutation in *ECR*. Cutinanalysis of *cer10* exhibited a reduction in α-hydroxy fatty acids and a slight accumulation in VLCFAs. Analysis of the expression of α-hydroxylases did not reveal the enzyme that is responsible for the α-hydroxylation in the cutin monomers. The analysis of lipids of the seed coat of *cer10/ecr* showed a primary effect of the lack of *ECR* as lipids with longer chain lengths are reduced when compared to the wild type but the elongation of VLCFAs in *cer10* is not completely abolished.

In another project the organ fusion mutant *cer13* was characterised. In an approach to map the *cer13* gene the map based cloning strategy was chosen and the analysis points to a specific location on chromosome 3 but further fine mapping strategies will be needed to identify *CER13*.

The method of cutin analysis was applied to *O.sativa* japonica and the cutin composition of rice has been established. In the future it will be possible to analyse putative cutin mutants in rice to identify components of the cutin biosynthesis in *O.sativa*.

The experiments in this thesis investigated the composition of cutin in Arabidopsis. The application of this method will lead to an understanding of the cutin biosynthesis and its involvement in signalling and plant protection. Still further studies on other plant species will be necessary as the cutin composition of Arabidopsis is different than in other plants and the biosynthesis of cutin components in Arabidopsis might not be true for all land plants.

6. Zusammenfassung

Pflanzliche Oberflächen sind so konzipiert, daß sie die Pflanze optimal schützen. Die Schutzschicht auf der Epidermis besteht aus verschiedenen Komponenten: der Zellwand, der Cuticula und den Wachsen. Die Cuticula ist aus Cutin und Cutan zusammengesetzt, Wachse sind entweder eingelagert (intracuticuläre Wachse) oder bilden auf der Oberfläche Kristalle (epicuticuläre Wachse). Die Zusammensetzung von Cutin und Wachs ist in vielen Pflanzen bekannt, und es gibt Modelle für die Biosynthese der langkettigen Komponenten. Allerdings konnten noch nicht viele Enzyme, die an diesen Stoffwechselwegen beteiligt sind, identifiziert werden. Generell wird angenommen, daß eine defekte Cutinschicht mit Organfusionen bei Mutanten in Zusammenhang steht. In der Modellpflanze Arabidopsis thaliana sind einige Fusionsmutanten bekannt; der Bezug zum Biosyntheseweg der Cutinschicht konnte bisher allerdings nicht hergestellt werden, weil es noch keine Methode zur Analyse der sehr dünnen Cutinschicht in Arabidopsis gab.

Mögliche Analysemethoden wurden in dieser Arbeit auf ihre Anwendung in Arabidopsis getestet und auf verschiedene putative Mutanten mit Defekt in der Cutinbiosynthese angewendet. Es wurde die Methode der Komplettextraktion von Arabidopsis-Blättern verwendet mit anschließender Depolymerisierung durch methanolische Salzsäure, die sich als effektiver erwies als die Depolymerisierung mit BF_3/Methanol. Die Extraktion der lipophilen Bestandteile erwies sich mit Hexan als optimaler als eine Extraktion mit Chloroform und die Analyse der Monomere wurde mittels GC-MS bzw. mit GC-FID durchgeführt. Die Analyse zeigte, daß das Cutin von Arabidopsis eine von anderen Pflanzen unterschiedliche Zusammensetzung hat. Es besteht hauptsächlich aus gesättigten und ungesättigten α- bzw. ω-hydroxilierten Fettsäuren, Disäuren, langkettigen Fettsäuren und Alkoholen. Die Zusammensetzung des Cutins von verschiedenen Mutanten ergab aufschlußreiche Ergebnisse.

Das Cutin von *ace/hth* bestätigte vorhergehende experimentelle Daten, denn es zeigte eine Akkumulation von ω-Hydroxy-Fettsäuren und eine leichte Abnahme

der Disäuren. Da bekannt ist, daß *ace/hth* epidermisspezifisch exprimiert wird und die Analyse von allen löslichen Lipiden ergab, daß ω-Oxo-Fettsäuren in kleineren Mengen vorliegen und ω-Hydroxy-Fettsäuren akkumulieren, ist ACE/HTH ein Kandidat der verantwortlich ist für die Oxidation von ω-Hydroxy-Fettsäuren zu ω-Oxo-Fettsäuren, die dann zum Einbau in das Cutin zu Disäuren weiteroxidiert werden.

Eine weitere Organfusions-Mutante, bei der die Cutinanalyse zu einer Aufklärung der Funktion beitrug, ist bodyguard (bdg), ein Enzym mit Homologie zu α/β Hydrolasen. Eine Anreicherung aller Cutinkomponenten, ebenso wie der epicuticulären Wachse, auf das 2-3fache konnte in dieser Mutante gemessen werden. SEM-Bilder zeigten eine dickere, aber weniger dichte Cuitula abwechselnd mit Abschnitten ganz ohne eine Cuticula. All diese Aspekte führten zu dem Schluß, daß BDG eine Rolle bei der Veresterung der Cutinbestandteile spielen könnte, da das Cutin von *bdg* nicht korrekt verestert ist und die Pflanze versucht, diesen Defekt durch eine Akkumulation von Cutinmonomeren und Wachsen auszugleichen.

Die Cutinanalyse weiterer Mutanten und transgenen Pflanzen zeigte zwar Unterschiede in ihrer Cutinzusammensetzung, konnte allerdings nicht zur Funktionsaufklärung der Gene beitragen. Hierfür sind bei *pel3*, *cer13* und FDH::FAE noch ergänzende Analysen nötig, da die Cutinanalysen unspezifische Veränderungen der Zusammensetzung aufweisen.

Desweiteren wurde *cer10/ecr* charakterisiert, denn CER10/ECR ist vermutlich verantwortlich für den letzten Schritt der Elongation der langkettigen Fettsäuren im FAE Komplex auf der cytosolischen Seite des ER. Zu den morphologischen Defekten von *cer10/ecr* zählen veränderte Größe, fusionierte Blüten, reduzierte Wachsmengen auf allen oberirdischen Organen, veränderte Oberfläche der Sepale und gelegentliche Organduplikationen in der Blüte. Es konnte nachgewiesen werden, daß CER10 allelisch ist mit dem publizierten ECR Gen. Somit müssen sich alle Phenotypen auf den Defekt in ECR zurückführen lassen. Die Cutinanalyse von *cer10/ecr* zeigte eine Reduktion der α-Hydroxy Fettsäuren und eine leichte Akkumulation der langkettigen Fettsäuren. Die Expressionsanalyse von putativen α-

Hydroxylasen ergab allerdings noch keinen Aufschluß, welches Enzym dafür verantwortlich sein könnte oder wie ein Defekt in *ECR/CER10* dazu führt, daß weniger α-Hydroxy Fettsäuren im Cutin eingebaut werden. Die Analyse der Lipide der Samenschale wies eindeutiger auf den Verlust der ECR-Funktion hin, denn hier waren alle längerkettigen Lipide in geringeren Mengen vorhanden als im Wildtyp. Allerdings ist die Elongation von langkettigen Fettsäuren in *cer10/ecr* nicht vollständig unterbunden.

Im dritten Teil der Arbeit wurde *cer13* charakterisiert. Die Klonierung von *CER13* wurde durch Eingrenzung des Lokus auf Chromosom III mittels „map based cloning“ durchgeführt. Allerdings weisen die morphologischen Veränderungen, die biochemische Characterisierung der Cutinzusammensetzung und die biochemische Analyse der Lipide der Samenschale nicht auf die Funktion von CER13 hin und weiteres Klonieren wird nötig sein.

Durch die Applikation der hier angewendeten Cutinanalyse auf Oryza sativa konnte die Cutinzusammensetzung der Unterart Japonica analysiert werden und es wird möglich sein, verschiedene Reismutanten mit defektem Cutin zu analysieren.

Mit den Experimenten in dieser Arbeit untersuchte ich die Zusammensetzung von Cutin in Arabidopsis. Die Anwendung dieser Methode wird zum Verständnis der Cutinbiosynthese, der Funktion von Cutinmonomeren in Signalprozessen und dem Schutz der Pflanze vor Pathogenen durch die Cuticula beitragen. Die Analyse und Übertragung des Wissens von Arabidopsis wird allerdings limitiert sein, da das Arabidopsis Cutin ein anderes ist als in den Landpflanzen, in denen es bisher analysiert wurde.

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